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THE ROLE OF POLYSACCHARIDES IN *PLASMODIUM FALCIPARUM* MALARIA

Sharon Rovelli

A dissertation submitted to the Faculty of Medicine, University of the Witwatersrand, in fulfillment of the requirements for the degree of Master of Science in Medicine.

Johannesburg, 1998

DECLARATION

I, Sharon Rovelli declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.



11TH day of NOVEMBER, 1998.

PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS STUDY

PUBLICATIONS

The effect of curdlan sulphate on *in vitro* growth of *Plasmodium falciparum.* **Transactions of the Royal Society of Tropical Medicine and Hygiene** 1994; 88: 686 - 687.

PRESENTATIONS

S Rovelli, K Masek, I Havlik, B Herweg.
The effect of lentinan on monocytes and *Plasmodium falciparum* infected erythrocytes.
Proceedings of the 26th Annual SA Pharmacological Society Congress - Bloemfontein.
13 - 15 September 1992.

S Rovelli, K Masek, I Havlik.
The effect of lentinan stimulated monocytes on *Plasmodium falciparum* survival.
Proceedings of the 22nd Congress of the Parasitological Society of Southern Africa - St. Lucia.
28 June - 2 July 1993.

S Rovelli, K Masek , I Havlik.
The effect of curdlan sulphate on the survival of *Plasmodium falciparum in vitro.*
Proceedings of the 27th Annual SA Pharmacological Society Congress - Karos Lodge.
19 - 22 September 1993.

K Masek, S Rovelli, Z Wu, I Havlik.
The effect of the immunostimulating drug lentinan in *Plasmodium berghei* infection in mice.
Proceedings of the 27th Annual SA Pharmacological Society Congress - Karos Lodge.
19 - 22 September 1993.

S Rovelli, I Havlik, K Masek, * Ajinomoto Co.
The *in vitro* effect of curdlan sulphate in combination with quinine and chloroquine on *Plasmodium falciparum.*
Proceedings of the XIIth International Congress of Pharmacology - Montreal, Canada.
24 - 29 July 1994.

C Brown, S Rovelli, S Evans, I Havlik.
The effect of curdlan sulphate on *Plasmodium berghei* infection in Balb/c mice.
Proceedings of the 28th Annual SA Pharmacological Society Congress - Cape Town.
22 - 24 September 1994.

ABSTRACT

Malarial infections involving *P. falciparum* are becoming increasingly difficult to treat with conventional antimalarials due to the emergence of drug resistant strains and the high level of toxicity associated with compounds currently in use. Novel antimalarial therapies are therefore in great demand. Two ß-1,3-D-glucans, lentinan and curdlan sulphate were investigated as potential therapeutic candidates .

Lentinan is an immunomodulating polysaccharide which is able to augment the body's natural response to cancer as well as various kinds of parasitic, bacterial and viral infections including AIDS. Lentinan's immunologic effects are host-dependant, mediated indirectly through both monocytes and cytotoxic T cells. The direct effect of lentinan on *P. falciparum* and the effect of monocyte supernatants from monocytes stimulated with parasite culture medium, parasites and Lentinan at varying concentrations, were assessed using the tritiated hypoxanthine uptake method. Monocytes were isolated from human peripheral blood according to the modified method of Boyum. Lentinan was found to exert no direct effect on *P. falciparum in vitro* and had no stimulatory effect on monocytes *in vitro*. The next phase of experimentation i.e. the bridge between *in vitro* and *in vivo* work, determined the effect on *P. falciparum* of supernatants from rabbit monocytes stimulated *in vivo* with 1mg/kg lentinan. There was no difference in percent parasite survival *in vitro* when supernatants from the rabbit monocytes stimulated *in vivo* with lentinan compared to the control group were tested. The final step in experimentation dealt with the *in vivo* effect of lentinan at 10mg/kg/day on the clinical course and outcome of *Plasmodium berghei* infection in Balb/c mice. Lentinan was ineffective in clearing parasitaemia, and had no effect on body mass, spleen index or haematocrit when compared to the control animals.

Since Lentinan is ineffective both *in vitro* and *in vivo,* it does not appear to be an effective candidate for treatment in acute *Plasmodium* infection.

Curdlan Sulphate (CRDS), has exhibited blocking effects on the binding of HIV-1 virions to the surfaces of target cells *in vitro* without the toxicity exhibited by other sulphated polysaccharides. It is proposed that this compound may similarly inhibit the binding to, and subsequent invasion of erythrocytes by *P. falciparum* merozoites. Drug sensitivity tests were carried out on *P. falciparum*

in vitro, using the uptake of radiolabelled tritiated hypoxanthine [^{3}H], to determine the IC_{50} values of CRDS over one and two cycles of parasite growth. Combination experiments with the aminoquinoline antimalarials: quinine, chloroquine and mefloquine, were then carried out on both the chloroquine-sensitive (3D7) and chloroquine-resistant (FCR3) strains of *P. falciparum.* No IC_{50} was obtained for the first cycle of growth since merozoite reinvasion of erythrocytes occurs only in the second cycle. The IC_{50} value here was found to be 4.5 ± 1.2 μg/ml , which is very similar to the concentration of CRDS which inhibits HIV-1 infection *in vitro.* All the combination experiments indicated a synergistic effect between the CRDS and the quinoline compounds tested.The *in vivo* effect of CRDS on the clinical course and outcome of *P.berghei* infected Balb/c mice was then determined. A dose response curve was developed using a wide range of CRDS concentrations administered subcutaneously to *P.berghei* infected mice. CRDS was successful in inhibiting parasite multiplication *in vivo.* The IC_{50} *in vivo* was estimated at 25mg/kg/day.

These positive results both *in vitro* and *in vivo*, indicate that CRDS may be a potentially excellent candidate for adjunct therapy with the classical antimalarials.

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1. CHAPTER ONE - GENERAL INTRODUCTION

Not long ago, the world seemed poised for victory in its age old struggle against malaria. Armed with new drugs and potent pesticides such as DDT, the World Health Organization (WHO) declared in 1955 that the disease could be eradicated. But today, the number of cases in many parts of the world is rising and we are actually worse off now than we were in 1950 (Cowley, 1994).

Over two billion people, half of the world's population are exposed to the disease, about 200 million people acquire new infections each year, and the most severe form of human malaria caused by one of the four species of malarial parasite, *Plasmodium falciparum*, directly results in over two million deaths per year (Peters W, 1990).

Since most of the deaths in malaria are caused through complications arising from cerebral malaria, the next two sections will elaborate on the role of cytoadherence and sequestration, as well as the role of cytokines, in the disease.

1.1 THE SYMPTOMS ASSOCIATED WITH SEVERE MALARIA INFECTION

Severe malaria is characterised by a high percentage of parasitised erythrocytes (usually > 5%) and a wide range of pathological processes which involve the renal, pulmonary and neurological systems. Anaemia, metabolic acidosis and hypoglycaemia are all serious complications associated with the disease (Campbell, 1991). The destruction of parasitised erythrocytes, the destruction of uninfected erythrocytes by the spleen and the suppression of erythrocyte production are all causes of anaemia. Acidosis results from impaired renal function and lactate generation by parasite metabolism.

Hypoglycaemia is caused by parasite depletion of available energy sources and is also compounded by the anorexia and vomiting associated with an acute malarial attack.

1.2 CYTOADHERENCE AND SEQUESTRATION IN SEVERE/CEREBRAL MALARIA

In defining the critical pathology that leads to death from infection with *P. falciparum*, much attention has been focussed on the catastrophic syndrome referred to as cerebral malaria. Recent studies on the pathophysiology and treatment of cerebral malaria in Africa have suggested that it can be a rapidly evolving neurologic disease, where the progression from disease-free to full-blown cerebral malaria and even death can occur within 36-48 hours (Phillips *et al.*, 1990). Traditionally, cerebral malaria has been diagnosed in patients with impaired consciousness or persistent coma, when parasites are detected in the blood (Phillips *et al.*, 1990). The WHO (1986), has restricted the definition of cerebral malaria to being "Severe and complicated malaria, frequently of sudden onset with a convulsion, followed by persisting unconsciousness". Blumberg *et al.*(1996) however, recommend extension of the definition to include: "Disturbed consciousness, confusion or convulsions not explained by hypoglycaemia or other metabolic abnormality".

Cerebral malaria is believed to be a result of the sequestration of mature *P. falciparum* infected erythrocytes in the small cerebral blood vessels, causing a blockage and leading to focal anoxia in the brain tissue (WHO Malaria Action Program, 1986). This sequestration also occurs in the postcapillary venules of many other organs and tissues in the body and is crucial to parasite survival because it prevents destruction in the liver and spleen (Biggs *et al.*, 1989). In the early stages of invasion, the parasitised erythrocytes flow freely through the blood vessels and capillaries. As the parasites mature, knob-like protrusions begin to develop on the surface of the erythrocyte membrane and the parasitised erythrocytes become "lodged" in, and marginated along, the venous

walls, in close contact with the endothelial lining (Barnwell, 1989). Not only do the infected erythrocytes interact with the endothelial lining, but they also interact with each other. Consequently, the number of circulating parasites is seen to decrease.

Several studies have been carried out in order to determine whether or not the knobs which develop on the erythrocyte membrane are essential for cytoadherence to take place. Biggs *et al.*(1989) showed that a knobless clone of *P. falciparum* was able to cytoadhere to C32 melanoma cells *in vitro* (sequestration has been modelled *in vitro* in the laboratory using endothelial or melanoma cell models since they produce a similar pattern of cytoadherence for different *P. falciparum* isolates). However, although these and other results indicate that parasite-derived changes in the erythrocyte membrane (including the insertion of new or altered proteins referred to as parasite-induced adhesion ligands) can be expressed on the infected red blood cell membrane independant of knob formation (Molyneux, 1990), there is no indication that the knobs are **not** necessary for the cytoadherence of parasitised erythrocytes to endothelial cells *in vivo*. Thus, the possibility cannot be ruled out that there is more than one mechanism operating in cytoadherence, and molecular biology is now aimed at determining exactly which adhesion ligands and receptors are involved in this process.

1.2.1 THE MOLECULAR BASIS OF CYTOADHERENCE *IN VITRO*

A number of parasite proteins have been proposed as possible candidates for the cytoadherence ligand. The most likely one to date has been identified by Leech *et al.*(1984) as Pf EMP-1. Pf EMP-1 was present on four knobby, cytoadherent strains of *P. falciparum*, but was not present on their knobless, noncytoadherent counterparts. Other proteins, for example MESA (EMP-2), 11.1 gene product and TRAP, have also been proposed to be the adhesion ligand. They are unlikely candidates

however, since they do not bind to the C32 melanoma or endothelial *in vitro* cytoadherence model cells, as Pf EMP-1 does.

As far as host cell receptors for the cytoadherence of erythrocytes are concerned, five potential receptors have been identified : CD36 (Barnwell *et al.*,1985), intercellular adhesion molecule 1 (Berendt *et al.*,1990) and thrombospondin (Roberts *et al.*,1985. More recently a role for the vascular adhesion molecule and E-selectin have been implied (Ockenhouse *et al.*,1992). CD36 is an 88 -kDa membrane glycoprotein, which has been shown to competitively inhibit the binding of parasitised erythrocytes to melanoma and endothelial cells when present in its purified form. Intercellular adhesion molecule 1 (ICAM-1) is a variably glycosylated glycoprotein in the same transient expression system as CD36, but these results still have to be confirmed with wild parasite isolates. Thrombospondin (TSP) is a large trimeric, multifunctional glycoprotein, capable of binding infected red blood cells (Asch *et al.*,1987) and capable of binding to CD36. One suggestion is that the adhesion between melanoma or endothelial cells and infected erythrocytes, might involve a complex of CD36, TSP and the infected erythrocyte ligand. On the other hand, studies carried out by Barnwell *et al.*(1989) showed firstly, that infected erythrocytes are able to bind to CD36 in the absence of TSP and secondly, that adherence to TSP is calcium dependant but adherence to CD36 is calcium independant. These results indicate that the infected erythrocytes may bind to CD36 and TSP independantly and that cytoadherence may occur primarily through the interaction of the CD36 receptor and the parasite-induced ligand. They may also indicate that different ligands are responsible for adhesion of erythrocytes to CD36 and TSP.

In any case, there are still several unanswered questions with regards to cytoadherence. One important question is - which of the receptors identified *in vitro* are relevant to sequestration *in vivo*? Perhaps CD36 mediates margination of parasitised cells through adhesion to the vascular endothelium and TSP aids the aggregation and layering of infected cells (Barnwell, 1989) *in vivo*. It

is quite possible that additional, but as yet unidentified, receptors and ligands are also involved in sequestration.

Another very relevant question about sequestration, involves determining the relationship between cerebral cytoadherence and the development of cerebral malaria. Cerebral malaria has a sudden onset and occurs in only a small number of usually non-immune people infected with *P. falciparum* (Phillips and Solomon, 1990). Since sequestration of erythrocytes in cerebral vessels is a general occurrence in falciparum malaria, other factors besides selective sequestration must mediate the onset of cerebral malaria.

Another adhesion property demonstrated by some *P. falciparum* isolates, associated with severe malaria, and possibly a contributor to the phenomenon of sequestration, is rosette formation. Rosetting occurs when uninfected erythrocytes bind spontaneously to erythrocytes infected with mature asexual parasites (David *et al.*, 1988). Studies in the Gambia have shown that children afflicted with cerebral malaria have a higher mean rosette frequency than those children with mild malaria (Carlson *et al.*,1990). These results suggest that rosetting may contribute to the pathogenesis of cerebral malaria, possibly by obstruction of blood flow in the microvasculature (Rowe *et al.*,1995). Recently, however, Al-Yaman *et al.*(1995), found that there was a lack of significant association between erythrocyte rosetting and disease severity in children in Papua New Guinea. The difference in results compared to the Gambian study may indicate a geographical variation in parasite expression of rosette ligands, in host cell receptors on uninfected red cells, or variations in aspects of the immune response to infection (Al-Yaman *et al.*,1995). At this point in time, it therefore remains unclear, whether rosetting itself is important in the pathogenesis of severe malaria , or if it is simply a marker for some other factor which mediates the disease process.

Clark *et al.*(1989) have suggested that monokines and lymphokines may play an important role in mediating malaria pathology. They may act by increasing the adhesiveness of parasitised

erythrocytes (which could possibly influence rosette formation), increasing the adhesiveness of endothelial cells, or they may even have a direct effect on the activity of the brain.

1.2.2 CYTOKINES AND MALARIA

The pathophysiological response to infection with *P. falciparum* is highly variable amongst individuals and depends on several factors, one of which is previously acquired immunity. Studies on the effects of *Plasmodium* infection on the central nervous system in mice and more recently in humans, have also revealed that severe disease is associated with a marked increase in several cytokines such as interleukin-1(IL-1), interleukin-6 (IL-6), γ-interferon (γ-IFN), and most notably, tumour necrosis factor (TNF) (Grau *et al.*, 1989).

Since manifestations of *P. falciparum* infection including hypoglycaemia, coma, and a rapidly progressive clinical course, are similar to those of diseases in which cytokines mediate immunopathology (one example being septic shock or endotoxemia), secretion of these mediators is thought to be responsible for many of the clinical features of malaria. The evidence that increased concentrations of cytokines are associated with severe *P. falciparum* infections, suggests that cerebral malaria results from a pathological immune response. In other words, IL-1, IL-2, TNF and γ-IFN are conventional immunoregulatory molecules in physiological concentrations but they evidently mediate systemic toxicity when their production gets out of hand.

Although the intricate connections of the cytokine network operating in human malaria have yet to be disentangled, I will attempt to summarise the most important monokines and lymphokines involved in malarial pathology.

T cells and the B cells are respectively involved in cell mediated and antibody mediated responses in an individual upon infection with a foreign organism. T-cells can be grouped according to the

cytokines they release. The helper T cells, T_H1, secrete the inflammatory cytokines interleukin-2 (IL-2), and γ interferon (γ-IFN); while the T_H2 cells secrete the antibody helper cytokines IL-4, IL-5, IL-6 and also IL-10. The former are responsible for activating macrophages, which are able to eliminate intracellular parasites by phagocytosis and the release of toxic molecules such as nitric oxide (NO), reactive oxygen intermediates (ROI), proteolytic enzymes and lipid mediators; whereas the latter act as growth factors for B cells and in so doing, stimulate the production of antibodies by the immune system (Taverne, 1993).

Macrophages which are activated by γ-IFN (which is reported to be present in the serum of patients with falciparum malaria), are not only primed to release noxious amounts of ROI and NO, but they are also stimlulated to secrete IL-1 and TNF. IL-1 has been shown to stimulate hepatocytes to produce acute phase proteins in malaria infection (Gillespie *et al.*, 1991) and both TNF and IL-1 are endogenous pyrogens and thus cause fever (Clark , 1987). TNF is in turn able to induce the production of IL-1 and vice-versa (Nedwin *et al.*, 1985).

The increased production of TNF by stimulated macrophages can serve to either help or harm the host. Although moderate levels of TNF in *Plasmodium falciparum* infection may be beneficial to the host, by resulting in increased killing by neutrophils of erythrocytic forms of the parasite as well as merozoites (Kumaratilake and Ferrante, 1995), research and attention has primarily been focused on the role that excessive amounts of TNF play in contributing to the pathology of malaria.

Since the advent of recombinant TNF, evidence for its involvement in malarial pathology is accumulating rapidly. Clark *et al.*, (1987) made an interesting discovery - when 10μg of recombinant TNF was injected into mice with low parasitemias of *P. vinckei*, rapid changes, including liver damage, pulmonary neutrophil margination and hypoglycaemia, were produced, as seen in the

terminal illness. The same dose injected into healthy mice however, had a negligible effect. Toxicity trials carried out in human volunteers using pharmacological doses of TNF, produced the same symptoms i.e. fever, chills or rigors, headaches, myalgia, nausea and vomiting as in severe malaria infection (Phillips and Solomon, 1990). Unfortunately, these *in vivo* experiments cannot determine whether TNF causes these changes directly, or by the initiation of other mediators. As discussed above, TNF is functionally interwoven with interleukins and interferons, sharing or reinforcing various activities. Whether these other mediators work through TNF or whether TNF works through them, is yet to be determined.

Another of the many functions of TNF, its capacity to influence endothelial cells, is an important factor in defining its role in the pathology of malaria. TNF acting synergistically with IL-2, enhances human endothelial cell procoagulant activity (Nawroth and Stern, 1986), alters the cell morphology (Sato *et al.*, 1986) and also makes them adhesive for neutrophils (Pohlman *et al.*, 1986). Therefore, by circulating during an attack of malaria, TNF is hypothesised to make endothelial cells more adhesive for the knobs on the parasitised erythrocytes, perhaps through endothelial projections which are particularly prone to form in cerebral vessels (Clark, 1987), thus promoting sequestration.
It has also been suggested (Berendt *et al.*,1990) that TNF might promote cytoadherence in man because it has been shown to increase the expression of surface molecules such as ICAM-1 *in vitro*. However, any theory linking circulating TNF with cerebral malaria must account for low levels of TNF in some comatose patients, high levels in some healthy individuals and those with benign or asymptomatic malaria, and innate gender and genetic differences in the capacity of healthy individuals to produce TNF (McLaughlin *et al.*, 1990). Clark (1987) suggests that the tolerance exhibited by many individuals to parasite load acquired after several weeks of clinical malaria, may in fact reflect tolerance to TNF and other monokines.Phillips and Solomon (1990) do not believe that

clinical measurements of serum or *in vitro* levels of TNF will reveal the answers to the above questions, but suggest that an anti-TNF antibody could possibly shed some light in this regard.

While the pathophysiology of the central nervous system dysfunction in malaria remains to be fully elucidated, the possibility that cytoadherence triggers a catastrophic burst of deleterious immune mediators that can kill the patient in a matter of hours has important implications for the objectives of antimalarial therapy (Grau *et al.*, 1989). Recent laboratory studies provide real hope that we will eventually understand the molecular processes which cause severe malaria, but we still have to be very careful in our extrapolation of laboratory results to clinical cases.

In the meanwhile, we have no choice but to continue treating the disease with the drugs that have been developed in parallel with our understanding of the disease. The following section serves to discuss the antimalarial therapies currently in use for the prophylaxis and treatment of *P. falciparum* infection.

1.3 MALARIA THERAPIES

The emphasis is now on control, rather than eradication of the disease (Gomes, 1993). While many naturally occurring and synthetic compounds have been used to treat malaria infections, the major control strategy is through chemotherapy using the quininoline-containing antimalarials. This group of compounds has evolved from the structural modification of quinine and includes 4-aminoquinolone compounds such as chloroquine (the most widely used of all antimalarials) and the quinoline methanol, mefloquine (Cowman, 1995). Other useful antimalarial drugs include the dihydrofolate reductase (DHFR) inhibitors; phenanthrene methanols (e.g. halofantrine), sesquiterpene lactones such as artemisinin (Bray *et al.*, 1993) and various other drugs in

combination. Unfortunately, the malaria parasite has managed to develop widespread resistance to most of the compounds used for treatment

and prophylaxis of the disease. Drug resistance in malaria has been defined by the WHO (1965, 1973) as "the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to, or higher than, those usually recommended but within the limits of tolerance of the subject". Many mechanisms have been postulated for the development of antimalarial drug resistance including drug pressure, extensive use of subcurative doses, migration, and increased virulence of the resistant parasites (Björkman *et al.*,1990).

There are at least two principal forms of drug resistance in malaria. First, resistance to the inhibitors of parasite folate metabolism, which arose soon after introduction of the antifolate drugs and spread independantly from many different foci. Secondly, resistance to chloroquine which took much longer to develop and spread from only two foci (Bray *et al.*, 1993).

It has therefore become imperative not only to develop novel antimalarial compounds, but also to understand the mechanisms that the parasite uses to circumvent the lethal effects of the drugs. I will initially briefly explain the mechanisms of action and resistance to antimalarials currently in use with emphasis on the quinoline antimalarials, since these drugs were used in combination studies in my area of research. I will then proceed to discuss the progress which has been made in the development of new antimalarial compounds.

1.3.1 DRUGS FOR MALARIA PROPHYLAXIS AND TREATMENT

Malaria results when infective *Plasmodia* sporozoites are injected into the bloodstream by the female *Anopheles* mosquito.

The action of antimalarial drugs is either (i) to inhibit the maturation of the early (pre-erythrocytic, PE) stages of the parasites that develop from sporozoites within hepatocytes, or (ii) by blocking the parasite multiplication (the schizogonic cycle) within erythrocytes that commences with the maturation and release into the circulation of the PE forms called merozoites (daughter cells). The release of the merozoites from the erythrocytes causes the pathological changes associated with malaria, such as fever, haemolytic anaemia and sometimes in the case of *P. falciparum* infection, cerebral malaria and death.

1.3.1.1 Folate Antag nists

The dihydrofolate reductase (DHFR) inhibitors, pyrimethamine and proguanil, are currently the most important antifolate drugs used to treat resistant malaria. Both proguanil and pyrimethamine act on the PE stages of the malaria parasite and their mechanism of action is to inhibit enzymes of the protozoan folic acid biosynthesis pathway (Peters, 1990). The major cause of resistance in the malaria parasite, is therefore the development and accumulation of specific, single point mutations at the active sites of these target enzymes (the dihydrofolate reductase molecules) (Bray *et al.*, 1993). Pyrimethamine and sulphadoxine are often administered simultaneously . Their combined action is highly synergistic, since both compounds act sequentially inhibit different enzymes in the same metabolic pathway of the parasite (Wernsdorfer and Payne, 1991).

Proguanil is a prodrug which is converted by the liver into the active compound cycloguanil. Cycloguanil and pyrimethamine have been shown to act as competitive inhibitors of DHFR in the malaria parasite. This results in a reduction in folate biosynthesis, which in turn blocks DNA replication (due to the decrease in pyrimidine synthesis). There is also a decrease in the conversion of glycine to serine and a decrease in methionine synthesis (Cowman and Foote, 1990).

The sulphone and sulphonamide (sulphur) drugs act on the folic acid biosynthetic pathway of the malaria parasite by (i) either directly inhibiting the enzyme dihydropteroate synthase (DHPS) or (ii) a toxic product is synthesised from the sulpha drug which inhibits the next enzyme in the pathway, dihydrofolate synthase. Sulphadoxine is the most frequently used sulphonamide. The suggested mechanism of action of sulphadoxine is direct inhibition of DHPS, since it has been shown to be concentrated in *P. falciparum* infected erythrocytes (Foote and Cowman, 1994).

1.3.2 QUININE-LIKE ANTIMALARIALS

Peruvian Indians in South America chewed the bark of the Chinchona tree to combat the "bad fevers" often caused by attacks of malaria. In the 16th century, Jesuit priests became aware of the medicinal properties of this tree. It was then transported back to Spain and used to treat malaria throughout Europe (Cowman, 1995).The antimalarial activity of the Chinchona tree is specifically related to the chinchona alkaloid quinine which is present in the bark, but other alkaloid components such as quinidine, have also exhibited marked antimalarial activity. Quinine is the oldest of the blood schizontocidal antimalarials and has always been a relatively scarce commodity. Even though quinine is fairly toxic and possesses some undesirable side-effects including hypoglycaemia, hypotension, and occasionally, an increase in uterine contractions and fetal distress (Cook, 1989), it is the drug of choice for the emergency treatment of severe or complicated falciparum malaria due to its rapid antiparasitic action (Warhurst, 1987). Alternative drugs are however, urgently required. The 4-aminoquinolones : chloroquine, amodiaquine, mepacrine and sontaquine, are analogues of quinine which were synthesised in the 1930's and 40's. After the Second World War, chloroquine was the most commonly used antimalarial used for the treatment of severe *Plasmodium falciparum* infection in humans. Inevitably, this led to the development of widespread chloroquine resistance

which prompted the development of mefloquine and halofantrine in the United States by the Walter Reed Army Institute in the 1960's . Both compounds are structurally similar to quinine and appear to have a similar mode of action (Cowman, 1995). Mefloquine has an unusually prolonged half-life and is currently recommended for prophylaxis and therapy against chloroquine-resistant *P. falciparum*. Halofantrine has a short half-life and is presently restricted to therapy (Peters, 1990).

1.3.2.1 Mechanism of Action

Quinine, chloroquine and related quinoline ring antimalarials, have their major effect on the mature stages of the asexual life cycle of the parasite (i.e. the intraerythrocytic stages of pigment producing parasites) and are commonly referred to as "blood schizontocides" (Peters, 1990).

The rapidly multiplying parasites require nutrients which are obtained in part from the digestion of the infected host cell's haemoglobin. The haemoglobin is taken into the acid food vacuole of the parasite, and is rapidly degraded into amino acids. The haem portion of the molecule is left in the form of ferriprotoporphyrin IX (FPIX). Since FPIX is toxic and cannot be degraded by the parasite, it is converted within the food vacuole into haemozoin, a non-toxic, insoluble polymer of haem units, with a crystalline-like structure (Wellems, 1992). Hemozoin is also referred to as malarial pigment . Studies on the effect of drugs such as chloroquine and mefloquine, have shown that the first morphological changes that occur in the parasite are swelling of the food vacuole and vesiculation and accumulation of undigested haemoglobin. These changes suggest that the major action of these drugs is to block the function of the food vacuole (Fitch *et al.*, 1983). There have been a number of hypotheses to explain the action of chloroquine. The most likely mechanism is that the compound intereferes with a haem detoxification enzyme in the parasite, thereby inhibiting the sequestration of FPIX into haemozoin This results in the accumulation of toxic amounts of FPIX in the parasite (Wellems, 1992) and eventual death.

1.3.2.2 Mechanism of Resistance

Since chloroquine is the most important antimalarial used to combat human malaria, most of the research into drug resistance has been dedicated to determining the mechanisms by which malaria parasites (and in particular *P. falciparum*) are able to evade its toxic action. Although a great deal of information has been gained as far as chloroquine's mode of action is concerned, we are still uncertain as to exactly how it acts against malaria parasites at the molecular level (Peters, 1990). There is evidence to suggest that chloroquine-resistant parasites concentrate significantly less drug than their chloroquine-sensitive counterparts (Krogstad *et al.*, 1987), which suggests that the mode of action of the drug is independant of the mechanism of resistance to the drug. It is essential to bear in mind though, that the genetic heterogeneity of the parasite lines studied may influence the expression of the chloroquine resistant phenotype.

1.3.2.2.1 The rapid efflux hypothesis

Krogstad et al.(1987), have suggested that an energy dependant efflux mechanism transports chloroquine out of resistant parasites at a 40-50 times greater rate than in chloroquine sensitive parasites. A number of different drugs, including the Ca^{++}-channel blocker verapamil, have been shown to partially reverse resistance by inhibiting this efflux and by raising chloroquine concentrations in resistant strains. These findings indicate that it is unlikely that chloroquine resistance is related to mutations of the haem polymerase enzyme in the parasite. A mechanism analogous to the the multi-drug resistance (MDR) mechanism in mammalian tumour cells has been proposed to account for the rapid-efflux resistant phenotype in the malaria parasite (Endicott *et al.*, 1989). MDR in tumour cells involves the rapid efflux of several chemically distinct anti-tumour

agents, which is mediated by an ATP-dependant transport protein termed P-glycoprotein (Pedersen, 1995). The P-glycoproteins in mammalian cells are encoded by MDR genes which are usually amplified in MDR tumour cells with overexpression of the protein (Hait *et al.*,1992). The similarities in the chloroquine-resistance phenotype and MDR phenotype in mammalian tumour cells suggested that there may be a P-glycoprotein homologue in *P. falciparum*. The first gene isolated from *P. falciparum* was termed the pfmdr1 gene which encodes the protein Pgh1. Pgh1 has a typical structure shared by many members of this protein family including the P-glycoproteins. It is highly likely that Pgh1 is involved in regulation of the pH of the food vacuole in *P. falciparum* and mutations in the protein could affect this function and decrease the accumulation of weak base drugs such as chloroquine (Cowman *et al.*, 1994). It is also clear that there is a strong correlation between amplification of pfmdr1 and overexpression of the Pgh1 protein with mefloquine, halofantrine and in some cases quinine resistance. However, the molecular basis for this is not yet understood.

1.3.2.2.2 Reduced uptake as the basis for chloroquine resistance: The weakened proton pump hypothesis

Chloroquine concentrates several hundredfold more in malaria infected erythrocytes than it does in uninfected erythrocytes (Yayon *et al.*, 1985). This is most likely due to the compound's weak base properties that allow its accumulation in acidic vesicles such as the food vacuole (Homewood *et al.*,1972). At neutral pH, chloroquine is in its uncharged form and can diffuse freely through membranes into the cell. In acidic compartments such as the food vacuole (which has a pH of 5.2), the chloroquine becomes doubly protonated and membrane impermeant, and therefore the chloroquine accumulates. The amount of chloroquine accumulated, will be dependant on the pH gradient across the food vacuole membrane (Yayon *et al.*, 1985).

1.3.3 THE SESQUITERPENE LACTONES

Extracts from the wormwood plant Artemisia annua have been used for several centuries in China as a treatment for fevers and malaria. In 1971, the sesquiterpene lactone, artemisinin (qinghaosu, QHS) was isolated as the active ingredient responsible for the reputed medicinal action (Klayman, 1985). The compound has been used to successfully treat thousands of malaria patients in China, and it is effective against both chloroquine-sensitive and chloroquine-resistant Plasmodium falciparum. Efficacy is high even in areas with multi-drug resistant strains. Artemisinin has a unique mode of action and is a more rapidly acting schizonticide than quinine, but problems with recrudescence (treatment with this compound has to be supplemented with other antimalarials such as mefloquine, quinine, tetracycline or doxycycline) and adverse reactions are likely to limit its use in this way in the West (Cook, 1989). Derivatives of QHS: the water soluble dihydroartemisinin and sodium artesunate and the fat soluble artemether and arteether appear to be more potent than QHS itself. To date, dihydroartemisinin and artemether are being used for treatment of malaria. Sodium artesunate in combination with sulphadoxine has also been shown to rapidly restore consciousness to comatose patients afflicted with cerebral malaria and arteether is being used in Phase III trials (de Vries *et al.*,1996). The artimisinin derivatives are extremely well tolerated and significant adverse effects or signs of toxicity have not been reported in human patients treated with therapeutic doses.

In general, the treatment of malaria has been greatly improved by the use of artemisinin and its derivatives especially in areas where multi-drug resistant parasites pose a serious problem.

1.3.4 ANTIBIOTICS

Several broad spectrum antibiotics and antimicrobial agents including doxycycline, tetracycline, rifampin, clindamycin, erythromycin and chloramphenicol have demonstrated significant antimalarial

activity in vivo, at concentrations within the range known to be effective for the treatment of bacterial infections, and either alone or in combination with other drugs (Geary *et al.*, 1983) e.g. significant antiplasmodial activity has been demonstrated for combinations of (i) clindamycin and ciprafloxacin with quinolines and (ii) co-trimoxazole with sulphonamides (Cook, 1989). Although doxycycline and tetracycline have been used successfully in malaria management , their use is limited because they cannot be administered in pregnancy or during infancy. Antimalarial therapy with tetracycline also requires extended treatment for success.

The efficacy demonstrated by such a wide range of antibiotics in the treatment of malaria infection, indicates that further research should be conducted along these lines. Modification of these drugs to extend their plasma half-life, could result in more effective and useful antimalarial drugs. By further examining the mechanism of action of these drugs on *Plasmodium falciparum*, the parasite metabolic pathways susceptible to chemotherapeutic intervention may be further elucidated (Geary *et al.*,1983).

1.3.5 OTHER COMPOUNDS UNDER INVESTIGATION FOR ANTIMALARIAL ACTIVITY

Other agents which have been investigated for potential antimalarial activity include emetine, metronidazole (James, 1985) and the immunosuppressive drug, cyclosporin A, which exhibits anti-HIV-1 activity (Thali, 1995).

Investigations into the inhibitors of riboflavin metabolism are also underway since chronic riboflavin deficiency has been shown to exert an antimalarial effect (Dutta *et al.*,1985). The riboflavin analogue 10-(4'-chlorophenyl)-3- methylflavin and 5-deaza-riboflavin have both shown antimalarial activity in vitro against *P. falciparum* (Cowden *et al.*,1987). Chlorhexidine, a topical antiseptic, has

also been reported to show antiriboflavin activity and has demonstrated considerable potency against *P. falciparum* in vitro (Geary *et al.*,1983).

The anticancer agent actinomycin D, is an extremely potent antimalarial drug in vitro and has been shown to be effective *in vivo*. Unfortunately, the drug is too toxic for casual prophylaxis against malaria but it may have a rare application in the treatment of life-threatening cases of drug-resistant falciparum malaria.

Gutteridge (1989) discusses a new drug in development, hydroxynaphthoquinone, 566C80, which may be potentially useful as (i) a prophylactic agent, (ii) a blood schizonticide for treatment purposes and (iii) as an anti-relapse compound (a tissue schizonticide). The drug is a ubiquinone antagonist and is targeted to the mitochondrial respiratory chain of the malaria parasite where it blocks electron transport. This mode of action is unique in comparison to existing blood schizonticides and causal prophylactics. Experimentally, 566C80 has been found to be active against all strains of malaria resistant to existing drugs including multi-drug resistant strains. Pre-clinical toxilogical studies also indicate that the compound has a potentially large therapeutic window, is orally bioavailable and metabolically stable in man and has a plasma half-life of approximately three days.

1.3.6 VACCINE DEVELOPMENT

Although the treatment of malaria is generally a much less contentious issue compared to prophylaxis, there is still considerable hope that a safe and effective vaccine will be produced in the near future.

The malaria parasite is able to evade attack from the host via several mechanisms: (i) poor immunogenicity of its surface antigens, (ii) antigenic variation, (iii) mutation and (iv) suppression of the immune response (Cook, 1989). Much work has been carried out with regards to the development of sporozoite, merozoite and gametocyte vaccines. The most progress however, has taken place in the

area of sporozoite vaccine development, where initial human trials have yielded encouraging, but not completely successful results (Patarroyo *et al.*, 1988). There is evidence to suggest that the cellular immune response to sporozoite antigens may be short-lived and possibly even be suppressed during an acute attack of *P. falciparum* malaria (Miller, 1988). A successful vaccine would therefore have to be stage-specific and contain antigens related to all three major stages of the parasite's life cycle.

Until a P. falciparum vaccine becomes a reality, much depends on the presently available chemoprophylactic and chemotherapeutic agents, together with the development of new compounds.

Two such compounds which I have investigated in this regard are the β-1,3-D-glucans, lentinan and curdlan sulphate. The following chapter explains the potential that these two novel compounds may have in the treatment of *Plasmodium falciparum* malaria infections.

1.4 THE ROLE OF THE ß-GLUCANS LENTINAN AND CURDLAN SULPHATE IN PLASMODIUM FALCIPARUM MALARIA

1.4.1 LENTINAN

There is reliable clinical evidence to support the existence of intrinsic resistance against cancer and other diseases in the human body. A drug which is able to boost the immune system or augment the body's natural defences to enhance this resistance, is a highly desirable commodity and would prove most beneficial in the fight against *P. falciparum.*

Much attention is presently being focused on immunotherapy as an alternative to conventional methods of disease control. Immunotherapy has its basis in Oriental medicine, the guiding principal

of which is the "maintenance of the natural balance of the human body and its restoration to balance during periods of ill health, rather than a direct assault on the disease" (Chihara, 1992).

Based on this concept, the immunopotentiating effects of a variety of compounds have been examined. Lentinan, a fully purified, high-order, structured, antitumour polysaccharide, first isolated in 1969 from the fruit body of the Japanese edible mushroom *Lentinus edodes*, is one of these. Antitumour polysaccharides have been isolated from diverse sources including higher plants, lichens, fungi, basidiomycetes and bacteria. They vary from glucan, mannan and hemicellulose to heteroglycan and lipopolysaccharide (Chihara *et al.*,1970). Lentinan is a β-1,3-glucan (Figure 1.1) composed only of glucose and has a relative molecular mass of 500 000 daltons. Lentinan's physical and chemical properties are precisely defined and its biological activity is reproducible. There is a significant correlation between the higher structure of lentinan and its biological function as a potentiator of both T-cell and non T-cell mediated responses (Maeda *et al.*,1988).

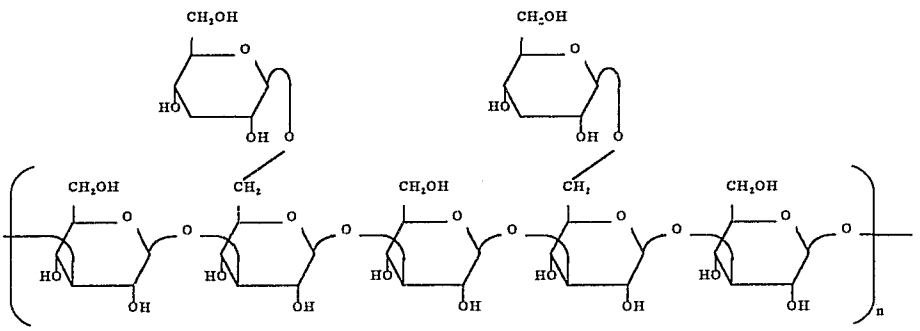

Figure 1.1 : The primary structure of lentinan, a β-1,6: β-1,3-D-glucan.

Lentinan is not directly cytotoxic against target cells and appears to act as a host defence potentiator (HDP). HDP's are agents which restore or augment the host-cell's responsiveness to lympho-cytokines, hormones and other bioactive factors by stimulating the maturation, differentiation and proliferation of crucial cells in host defence mechanisms (Chihara *et al.*,1989). The antitumor effect of lentinan is abolished in neonatally thymectomized mice (Maeda *et al.*,1971), decreased by the administration of anti-lymphocyte serum and inhibited by antimacrophage agents such as carra-geenin and silica (Chihara, 1992). Therefore, unlike other well known immunostimulants, Lentinan is a unique T-cell oriented immunopotentiator in which macrophage functions play an important part (Maeda and Chihara, 1971).

In order to evaluate the possible mechanisms of action of lentinan, it is necessary to briefly discuss the effect of lentinan on the various effector cells in the immune system.

According to Chihara *et al.*(1990), early reactions after lentinan administration occur as follows: there is an initial increase in various kinds of bioactive serum factors such as acute-phase protein inducing factor (APPIF), vascular dilation and haemorrhage inducing factor (VDHIF) and IL-3. These factors act on lymphocytes, hepatocytes and other cells, thereby accelerating various other biological reactions and immune responses in the host. In addition, lentinan has been shown to increase the activity of C? 'ıe third component of complement). According to Hamuro *et al.*(1980), "Since β-1,3-glucans possessing antigen presenting cell (APC) activating potency can generate active complement components such as C3b *in vivo*, and since C3b in turn is capable of rendering macrophages cytotoxic, the biological activity of β-1,3-glucans such as lentinan might be explained by their APC-activating effect". Several inflammatory responses in the host are also augmented, including increased susceptibility to histamine (Suga *et al.*,1986).

The infiltration of eosinophils, neutrophils, granulocytes and macrophages around target tissues is also a very important phenomenon induced by lentinan. Since macrophages act as accessory cells for both the cellular and humoral arms of the immune system, and as cytotoxic effector cells, they are a critical component of the immune system. Lately, evidence has also been reported concerning the role of the activated macrophage in the modulation of anti-tumour activity. It has been suggested that β-1,3-glucans may mediate their anti-tumour effect either by cytotoxic activation of macrophages, or by augmenting their accessory functions which include the "elaboration of monokines and the presentation of antigen, in the context of an appropriate immune-associated antigen, to 'T-helper cells" (Fruehauf *et al.*, 1982).

In this respect, interleukin-1 (IL-1 or lymphokine activating factor), is a monokine of particular interest since it facilitates antigen and lectin induced T-cell proliferation. IL-1 induces the production of IL-2 by T helper cells, as well as the production of Natural Killer (NK) Activity Factor and MAF. These factors amplify the maturation of immature (precursor) effector cells into cytotoxic T lymphocytes, NK cells and activated macrophages. Lentinan, therefore, not only augments the induction of antigen specific cytotoxic T-lymphocytes (CTL's), and antibody specific macrophage cytotoxicity (ADMC), but also augments non-specific cellular and humoral immune responses through NK cells and cytotoxic macrophages (Chihara *et al.*, 1990).

IL-1 is able to adequately replace macrophages in several T-cell proliferative systems, and its action can be diminished by steroids which leads to a strong inhibition of T-cell and B-cell responses. These responses may therefore be enhanced *in vivo*, if the production of IL-1 is boosted. It has therefore been proposed that one of the main effects of immunoadjuvants such as lentinan, could be to increase the production of IL-1.

As far as lentinan's involvement with T-cells is concerned, there is plenty evidence to suggest that lentinan preferentially enhances T-cell functions. Since *in vivo* induced T-cell mediated cytotoxic immune responses are very complex however, it has still not been established whether the ß-1,3 glucans interact with T-cells directly or not. According to unpublished work by Hamuro, there is evidence to suggest that ß-1,3 glucans act on T-cells via macrophages. Experiments have indicated that " ß-1,3 glucan-activated macrophages are depressed in their capability to secrete PGE_1. PGE_1 is in turn known to suppress the proliferation and cytotoxic effector function of CTL " (Hamuro *et al.*, 1978). When ß-1,3 glucans are added to an *in vitro* mixed lymphocyte culture, they are able to enhance the induction of alloreactive CTL responses. This finding will be of benefit in the analysis of the effect of ß-1,3 glucans on different cellular components involved in *in vitro* cytotoxic T-cell responses: eg. T-helper, T-pre-killer cells and macrophages.

The immunopotentiating effects of lentinan however, have been extensively researched *in vivo* as well - both in animal models and in human patients. A brief review of these studies is now in order:

1.4.1.1 Animal studies

As far as experimental study in animal models is concerned, the efficacy of lentinan was initially recognized by it's growth inhibiting activity against allogeneic sarcoma-180 transplanted subcutaneously in CD-1 mice. Complete regression of the sarcoma was observed after administration of lentinan at a dose of 1 mg/kg intraperitoneally daily for 10 days (Chihara *et al.*, 1970). Further investigations have elicited similar antitumour effects of lentinan on autochthonous and syngeneic tumour systems (Chihara *et al*, 1990).

Haba *et al.*,(1976) have investigated the selective suppression of T-cell activity in tumour bearing mice and its improvement by lentinan. It is well recognized that the immune response is often

suppressed in animals and humans which bear various tumours. Since specific immune responses to tumour-associated transplantation antigens have been demonstrated experimentally, the question is raised as to why immunogenic tumours escape from the host immune surveillance system. Previous studies have shown that mice which bear various transplantable tumours show a suppressed immune response to primary and secondary antigenic stimulations.

Although the mechanisms by which the host immune capabilities are suppressed in the tumour-bearing state may vary between the different tumour systems, experimentation by Haba *et al.*,(1976) on Ehrlich tumour bearing mice, attempted to clarify the mechanism of depressed immune surveillance in the tumour bearing host.

Their results showed that B-cell activity, as measured by the " anti-hapten antibody responses to hapten-conjugated thymus-independent carriers (TID) " was not impaired in tumour bearing animals, but T-cell activity, as measured by " the helper T-cell function on the induction of hapten-specific antibody responses to the hapten thymus-dependant carriers (TD) " (Haba *et al.*,1976), was markedly suppressed as compared to normal animals.

The suppression of T-cell activity, however, was almost com-pletely prevented by administration of lentinan. This ability of lentinan to prevent the suppression of T-cell activity in Ehrlich tumour-bearing animals appears to be a convincing experimental model system for the screening of prospective immunopotentiators especially relevant to the improvement of T-cell debility in tumour-bearing animals. Haba *et al.*(1976), suggest that treatment with lentinan may improve the trapping of T-lymphocytes or repopulation of damaged lymphoid organs in the tumour-bearing host, by these cells.

According to Chihara *et al.*,(1990), there are two important points concerning lentinan's mode of action in animal experimental models, and clinically in humans:

(I) Lentinan has no direct cytotoxicity (cytocidal or static) against tumour target cells (whether they are autochthonous or syngeneic tumours), and it's antitumour effect appears to be mediated by the host.

In order to determine whether or not lentinan and other ß-1,3 glucans are directly cytocidal against tumour cells, Sarcoma-180 tumour cells were cultured in medium containing various concentrations of the polysaccharides. The cells were 90-98% viable after twenty four hours indicating that these polysaccharides have no direct cytotoxicity against the tumour cells.

(ii) There exists a distinct optimal dose in the effect of lentinan (ie) the administration of 80 mg/kg/day in mice, does not produce as marked an effect as the administration of 1 mg/kg/day. Low doses appear to be much more effective than high doses.

The presence of an optimum dosage suggests that the mechanism of action of lentinan is in some way related to the immunological response of the host. Polysaccharides such as lentinan may play the role of interferon or interferon-inducer, because these substances behave similarly as part of the host's defence against tumours and viruses.

1.4.1.2 Studies in humans

Lentinan has also shown remarkable promise in the area of infectious disease in humans (Chihara *et al.*, 1990). The majority of immunosuppressed patients such as cancer or AIDS patients die of opportunistic infections. By treating these patients with HDP's such as lentinan, many of these infections have been circumvented.

1.4.1.3 Bacterial infections

Lentinan is able to increase host resistance against bacterial infections with *Mycobacterium*, *Staphylococcus*, *Pseudomonas* and *Escherichia coli*. In so doing, the patient is protected from sepsis, bacteraemia, pneumonitis and peritonitis. The compound also increases resistance to post operative fungal infections with *Candida albicans*.

1.4.1.4 Viral infections

Lentinan and related polysaccharides, also exhibit marked antiviral activity and increase host resistance to various kinds of viral infections, including the human immunodeficiency virus. When Lentinan was used in combination with azidothymidine (a drug exhibiting toxicity in most AIDS patients), the expression of the HIV surface antigen was more strongly suppressed than when azidothymidine was used alone. This combination therapy might become a prospective one against AIDS in the future since " any attempt to AIDS therapy must include not only suppression of viral replication, thereby halting the destruction of T_4cells, but also a strategy to enhance the immune system " (Chihara *et al.*, 1990).

1.4.1.5 Parasitic infections

As far as parasitic infections are concerned, it has been reported that host resistance against infection with malaria parasites for example depends on cell mediated immunity, and therefore a T-cell oriented adjuvant such as lentinan might be effective in the treatment of such parasitic infections.

1.4.1.6 Cancer Research

In the area of cancer research in human patients, lentinan has also shown much promise. In addition to being active against the cancer cells themselves, most chemotherapeutic agents are extremely cytotoxic to the cells involved in the immune response. As a result, cancer patients often die of secondary diseases and infections, such as pneumonitis. The body itself possesses intrinsic resistance to cancer and infectious diseases, and if this resistance is increased in some way, a major breakthrough in therapy against these diseases will ensue.

Lentinan has long been considered to be effective in the treatment of cancer patients and Lentinan therapy has shown very good results in prolonging life span. Lentinan is also a very safe compound, exhibiting only slight, transient side effects in human patients (Chihara *et al.*, 1990).

T-cell oriented immunoadjuvants such as lentinan, also play an important role in the immuno-therapy of cancer, and this role needs to be assessed. Usually, the bacille Calmette-Guérin (BCG) strain of *Mycobacterium bovis* is administered to the cancer patient as an adjuvant. However BCG adjuvant is associated with serious toxic side effects in these cancer patients.

Experimentation undertaken by Hamuro *et al.*(1978), attempted to compare the immune adjuvant activity of Lentinan with four other polysaccharides: Pachyman, Pachymaran, Carboxymethylpachymaran and Hydroxyethylpachyman. This activity was assessed according to their ability to induce alloreactive murine cytotoxic T-lymphocytes (CTL) in vitro (i.e. a T-cell mediated cytotoxic allograft response). The above five ß-1,3 glucans were all found to be effective as immune adjuvants in the mouse tumour system used without any resulting toxic side effects. Their results also indicated that in order for optimal augmentation of *in vivo* CTL responses to ur, lentinan had to be administered during the early phase of the immune response (i.e.) days 0, 1, and 2 after tumour challenge (in fact, suppressed cytotoxicity is observed if the glucans are administered during the late phase). The enhancing effect of Lentinan was also very much dose dependant.

Taguchi *et al.*(1985), carried out a randomized , controlled clinical study on the efficacy of lentinan in combination with tegafur for the treatment of inoperable and recurrent gastric cancer. The tegafur was either given alone or in combination with lentinan, and the efficacy of either treatment was evaluated according to various criteria:

(1) prolongation of patient lifespan,

(2) antitumour effects and

(3) side effects.

A statistical significance concerning lentinan's ability to prolong life span was observed. The lentinan plus tegafur group survived longer than the control group given oral tegafur alone.

As far as enhancement of antitumour effect by lentinan is concerned, a significantly greater effect was also observed in patients who had received the tegafur in combination with lentinan. This result elucidates a unique characteristic of lentinan for the treatment of cancer: In patients where cancer chemotherapy does not produce a complete or partial response, combined administration of lentinan could still result in a life - prolongation effect.

According to this three phase study on lentinan, adverse reactions were observed in only 32 out 469 patients. The toxic effects were mild and transient and included " eruption and redness (1.9%), feeling of mild pressure in the chest (1.7%), nausea and vomiting (1.7%), headache and a feeling of heaviness in the head (0.6%), hot sensation (0.6%), sweating (0.6%), fever (0.4%), flushing (0.4%), a decrease in white blood cell counts (0.4%), dizziness (0.2%), and a throat obstruction feeling (0.2%) " (Taguchi *et al.*, 1985).

Within the area of cancer research , Suzuki *et al.*(1990), revealed the possibility of using lentinan and IL-2 in combination for immunotherapy against cancer in order to reduce the detrimental side effects observed when large doses of recombinant IL-2 (r IL-2) are used alone. Combined administration of Lentinan and IL-2 was found to augment lymphokine - activated killer activity in vivo (endogenous LAK activity), in both normal and tumour bearing mice. Combination therapy

with Lentinan and IL-2 also showed significant prolongation of survival rate in patients.

" The augmentation of LAK precursor maturation by lentinan seems to be one of the possible mechanisms in the augmentation of LAK activity by lentinan administration " (Suzuki *et al*, 1990, p621). It is a well known fact that most tumour bearing hosts show a depression in both the production of and responsiveness to diverse lymphokines. Since lentinan is able to augment responsiveness of the host to lymphokines, this seems to be a rational approach to future immunotherapy against cancer.

Since this compound has proven to be only slightly toxic in certain human patients, and since it has been shown to stimulate host defence to various bacterial, viral and parasitic infections (Chihara *et al.*1990), it may become a " useful tool for revealing distinct interrelationships between immunity, inflammation and general host defence mechanisms" (Suga *et al.*, 1986).

1.4.2 CURDLAN SULPHATE (CRDS)

In recent years, much effort has been expended in seeking to elucidate the structure-activity relationships of antitumour polysaccharides. The structure and conformation of the (1→3/1β→6)-β-D-glucan lentinan has been studied extensively. Lentinan, with a branched β-(1→3)-linked glucan backbone and the linear polysaccharide Curdlan (Figure 1.2), have the same β-(1→3)-glucan backbone (Yoshida *et al.*, 1989).

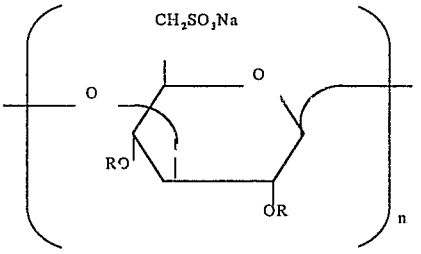

Figure 1.2: The chemical structure of curdlan sulphate (CRDS)

Curdlan is produced by a mutant strain (10C3K) of the bacterium *Alcaligenes faecalis* var *myxogenes* 10C3 (Kaneko *et al.*, 1990), and has a highly ordered structure as demonstrated by solid state nuclear magnetic resonance (NMR) studies. Initially, it was thought that the existence of ordered structures (i.e single and triple helices) and a high molecular weight were ssential for antitumour activity. However, Demleitner *et al.*(1992), found that some derivatives of curdlan showing antitumour activity, had no ordered structures. Other studies demonstrated that (1→3/1→6) -β-D-glucans from various Phytophthora species, with a low molecular weight and no helical conformation were active against the Sarcoma 180 (Demleitner *et al.*,1992). Therefore, it is not the highly ordered structures of branched (1→3)-β- D-glucans or a high molecular weight , but a (1→3)-linked backbone which appears to be advantageous for the antitumour activity of β- D-glucans.

Gerber *et al.*(1958), first reported that certain sulphated polysaccharides exhibit antivirus activities: i.e. agar and carrageenan were observed to inhibit influenza B and mumps viruses. In the following two decades, various other sulphated polysaccharides such as dextran sulphate, heparin and sulphated glycosaminoglycan , were found to inhibit viral replication of herpes simplex, polio and various other viruses (Kaneko *et al.*,1989). The emergence of AIDS in 1981 caused by HIV infection, led to the evaluation of sulphated polysaccharide compounds in antiviral activity against HIV. Carrageenan (Nakashima *et al.*, 1987) , heparin (Ito *et al.*, 1987), dextran sulphate (Baba *et al.*, 1988), fucoidan (Baba *et al.*,1988), pentosan polysulphate (Baba *et al.*, 1988) , polysulphated polyxylan (Biesert *et al.*,1988) and mannan sulphate (Ito *et al.*,1989) all showed anti-HIV activity. Sulphated polysaccharides extracted from marine algae are also reported to have anti-AIDS virus activity (Nakashima *et al.*,1989).

In addition to their role in HIV, sulphated polysaccharides are also considered to have potential as adjunct therapies in the treatment of severe malaria. Various sulphated polysaccharides including heparin, dextran sulphate, fucoidan (Butcher *et al.*, 1988) and most recently, curdlan sulphate (Havlik *et al.*,1994) have been shown to exhibit antimalarial effects. They are believed to be involved in the invasion of erythrocytes by malarial merozoites (Butcher *et al.*,1988), the invasion of sporozoites into hepatocytes (Pancake *et al.*, 1992) and in rosetting (Carlson *et al.*, 1992). In addition to their anti-parasitic activity, they may also have anti-disease activity as indicated by their role in the adhesion, activation and trafficking of leucocytes (Tyrrell *et al.*,1995)

The majority are unable to be utilized clinically in either HIV-1 or in malaria as therapeutic agents because of their toxicity or instability (Aoki *et al.*, 1991). Sulphated polysaccharides such as dextran sulphate have high blood anticoagulant activity, leading to undesirable side effects when used as

anti-AIDS drugs (Yoshida *et al.*, 1995). To date, heparin is the only sulphated polysaccharide to have been used clinically in the treatment of human cerebral malaria (Munir *et al.*, 1980). However, due to the severe side effects related to heparin induced bleeding, standard heparin is also no longer being used for this purpose.

A major advantage of CRDS over other sulphated polysaccharides for therapeutic applications is its very low toxicity and low blood anticoagulant activity. The anticoagulant activity of curdlan sulphate is lower (10 - 16 units/mg) than that of standard dextran sulphate (2 units/mg) (Yoshida *et al.*, 1995)or heparin (130 - 150 units/mg) (Kaneko *et al.*, 1989).

The anti-coagulant activity of curdlan sulphate *in* vitro was investigated by Evans *et al.*(1998) by studying the effect of the drug on cytoadherence (adhesion) to the CD36 receptor on platelet monolayers.The Curdlan was found to be ineffective in preventing parasitised erythrocytes from adhering to platelet monolayers, indicating that it does not have a significant anticoagulant effect.

Cytotoxicity of curdlan sulphate against HIV- uninfected MT-4 (a T4 lymphotropic cell line carrying human T-cell lymphotropic virus Type 1) cells was not observed at concentrations of up to 1000μg/ml (Yoshida *et al.*,1995) or even up to concentrations of 5000μg/ml (Kaneko *et al.*, 1989). In fact, curdlan sulphate completely inhibits HIV infection *in vitro* at a concentration of 3.3μg/ml (Yoshida *et al.*, 1995).

Also, no direct cytotoxicity of CRDS to H9 cells or H9/HIV-1 cells (no effect on cell viability or on cell growth characteristics) was observed *in vitro* in the course of continuous exposure to 100μg/ml CRDS for 2 weeks (Aoki *et al.*,1992).

In the area of malaria research, studies were carried out by Evans *et al.*(1998), who investigated the experimental conditions following exposure to curdlan under which new ring forms failed to appear. When parasites were exposed to CRDS at 50 μg/ml, no effect on the development of the parasite within the erythrocyte (i.e. through the ring, trophozoite and schizont stages) was observed . However, CRDS did delay schizont rupture and prevent merozoite invasion of new erythrocytes - indicated by a decrease in parasitaemia. This delay in schizont rupture was not due to irreversible damage, because when the curdlan was removed, the parasites recovered. Also, the merozoites produced by parasites which matured in the presence of CRDS, were viable and capable of successful invasion of new red blood cells. In another experiment, Evans *et al.*(1998) pre-incubated separate suspensions of merozoites and erythrocytes both with and without CRDS. The pre-treated cells were then washed and mixed together in different combinations. When either the erythrocytes or the merozoites which had been pre-treated were mixed together in any combination, invasion was inhibited (parasite survival was ~ 10%). This inhibition was not due to toxicity to the schizont infected erythrocyte, because curdlan also inhibited invasion of isolated merozoites. When neither the erythrocytes, nor the merozoites had been pre-treated with curdlan, parasite survival was 100%.

In vivo, studies by Kaneko *et al.*(1990) demonstrated that CRDS is not antigenic in guinea-pigs, shows no clinical side effects in mice and rats, and has no prominent anticoagulant activity in rats. No acute toxicity was observed following oral administration of curdlan at 10g/kg body weight to rats and mice (Kaneko *et al.*, 1992). As far as toxicity studies in human patients are concerned, it has recently been shown that CRDS produced dose related increases in CD4 lymphocytes in HIV infected patients after a 4 hour infusion and no clinical side effects were observed at any dose tested (Yoshida *et al.*, 1995).

The molecular weight (M.W) of CRDS is in the range of 2 x 10^4 to 25 x 10^4 daltons according to sulphation conditions, and sulphur content (S.C) has been in the range from 10.5% to 16.2% (Kaneko *et al.*, 1989). Structural differences seem not to be essential for anti-AIDs virus activities on HIV-1 and HIV-2, but the degree of sulphation (S.C) and molecular mass (M.W) of the Curdlan Sulphate are important (Yoshida *et al.*, 1995).

The half-life of curdlan sulphates in plasma have been found to depend on their molecular masses, being 60 and 180 min for molecular masses of 7 x 10^4 and 17 x 10^4 daltons, respectively (Kaneko *et al.*, 1990). These curdlan sulphates were transported to tissues such as liver, kidney, lymph node, and bone marrow within 1 hour, and remained in the tissues without degradation for 10 days (Yoshida *et al.*, 1995).

A great deal of research has been dedicated to uncovering the mechanism of action of sulphated polysaccharides. I will focus on the progress made as far as curdlan sulphate is concerned in both the areas of HIV and malaria.

The mechanism of action of sulphated polysaccharides against various types of viruses, is that they are either adsorbed onto the viral envelope or adsorbed onto the cell surface (Aoki *et al.*, 1991). In this manner, the binding to and/or penetration of target cells by the virus is blocked. Since the viral envelope does not have any contact mechanism to infect the cell surface, the result is complete inhibition of both viral replication and syncytium formation.

A study by Aoki *et al.*, (1990) suggested that in HIV, although CRDS acted on both the cell surface and the viral envelope to inhibit HIV-1 entrance into H9 cells, the compound was more effective on the viral envelope than on the cell surface. In contrast, other sulphated polysaccharides (i.e. dextran

sulphate, heparin and fucoidan) act predominantly on the CD4 receptor of host cells rather than on gp120 of the HIV-1 envelope. It is possible that the affinity of CRDS itself may be weaker for the cell surface CD4 receptor than the gp120 of the viral envelope. Most recently, Jagodzinski *et al.*(1996), have suggested that the anti-HIV activity of CRDS *in vitro* may be due to CRDS binding to the V3 region of gp120 on the HIV, thus interfering with the cell-virus membrane fusion process.

It has also been suggested that although CRDS possesses *in vitro* HIV-1 activity against HIV-1 virions which have already infected H9 ($CD4^+$ human cell line) cells, CRDS most likely does not disrupt the HIV-1 virions or work virucidally (Aoki *et al.*,1991). In a separate study however, Aoki *et al.*(1992) found that although endocytosed CRDS may have no effect on the integrated viral genome and/or provirus which may exist in a quiescent or inactive form, it may have antiviral activities involving inhibition of the reverse transcriptase of HIV-1 in the cytoplasm.

Aoki *et al.*(1991), have also discovered that short term treatment with CRDS (1 hour), showed relatively weak blocking effects on the binding of HIV-1 virions to the surface of H9 cells. In contrast, long term treatment with CRDS (2 weeks) was effective in inhibiting virus expression (determined by syncytium formation and levels of p24 antigens in soluble, intraviral, and intracellular forms). The most dramatic inhibition results were obtained when the compound was present both at the time of exposure of H9 cells to virus and during long -term follow-up treatment, but had no observable effect on virus expression in chronically infected (stable, previously infected) cells. CRDS therefore inhibits both the cell-free (represented by the establishment of new infections) and cell-associated (represented by stable infection) transmission of HIV-1 to host cells and interferes with early events in virus infection. In addition, it has been proposed that combined usage of CRDS with cell stimulating agents such as interleukins or

cytokines may shorten the latent period by provoking viral replication, thereby resulting in amplification of the effectiveness of the CRDS (Kaneko *et al.*, 1989).

As far as the mechanism of action of CRDS in malaria is concerned, Havlik *et al.* (1994), have recently demonstrated that curdlan sulphate has exhibits *in vitro* antimalarial activity against *Plasmodium falciparum,* by inhibiting merozoite invasion of the red blood cell. In order for the malaria parasite to be able invade the erythrocyte, there has to be an interaction between merozoite-associated receptors and specific ligands on the membrane of the erythrocyte. Ward *et al.* (1993), described the events which occur after the merozoite has invaded the erythrocyte as follows (Figure 1.3): maturation of the early ring forms of the parasite within the erythrocyte to form trophozoites then schizonts, release of merozoites, attachment of merozoites to uninfected erythrocytes, entry of merozoites into erythrocytes, and finally the formation of new ring forms. At this stage it is unknown whether the compound acts on free merozoites after their release or whether it binds to the membrane of uninfected erythrocytes, thereby blocking the entry of the merozoite into the erythrocyte.

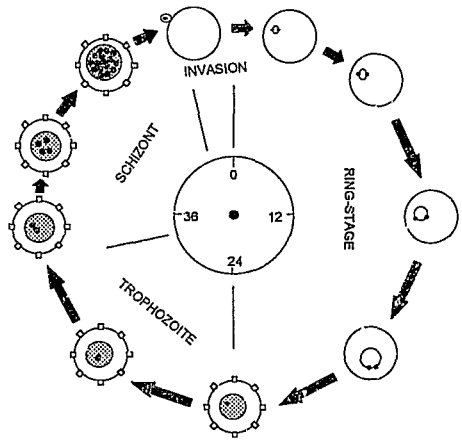

Figure 1.3 : The intraerythrocytic life cycle of the *P. falciparum* malaria parasite

In conclusion, the low toxic properties of CRDS demonstrate the safety and efficacy of this compound and suggest a promising usefulness for clinical applications in the treatment of HIV-1 infection (Aoki *et al.*, 1992). In addition, its marked anti-invasion activity on merozoites *in vitro*, without a marked anticoagulant effect , make curdlan a potential auxilliary compound in the treatment of severe malaria.

1.5 AIMS OF THIS RESEARCH

The two polysaccharides lentinan and curdlan have been used previously as successful adjuncts in the treatment of various infections and diseases including HIV. To date however, the effect of these compounds on parasitic (plasmodial) infections has yet to be established. This dissertation attempts to narrow this gap by focusing firstly on the effect of lentinan in its non-sulphated form on malaria infection both *in vitro* and *in vivo* and secondly by comparing this to the effect of curdlan in its sulphated form on malaria infection both *in vitro* and *in vivo*.

2. CHAPTER TWO - BASIC METHODOLOGY

2.1 MALARIA CULTURING *IN VITRO*

2.1.1 CULTURE MAINTENANCE

Long term *in vitro* culturing of the *Plasmodium falciparum* FCR-3 (chloroquine resistant) and 3D7-A (chloroquine sensitive) strains, was carried out according to a modified version of the methods established by Jensen and Trager (1976) and Freese *et al.*,(1988).

The parasites were maintained in continuous culture in human erythrocytes at 37°C, to which complete culture medium was added (section 2.1.1.1). The parasitised erythrocytes were maintained at a parasitaemia of approximately 5 - 10 % and a haematocrit of 5%. The 3D7-A strain was kept on an orbital shaker, to prevent the formation of gametocytes. This was not necessary for the FCR-3 strain. Each day at approximately the same time, spent culture medium had to be removed from the culturing flasks and replaced with fresh, pre-warmed complete culture medium. Parasite growth was assessed daily by preparing thin blood smears using a drop of blood from the bottom of the flask (section 2.1.1.4). Culturing was carried out in a laminar flow hood using sterile techniques (flaming and swabbing with 70% absolute alcohol).

2.1.1.1 Preparation of the complete culture medium

To 1L of autoclaved water: 10,4 g Roswell Park Memorial Institute (RPMI) medium, 5.94 g 4-(2-hydroxyethyl)-1-peperaxinethane sulphoric acid (HEPES) buffer, 4 g glucose, 44mg of hypoxanthine and 1ml of gentamicin sulphate (at 500mg/ml) was added, and stirred for one hour with an autoclaved

stirrer bar. This incomplete medium was filter sterilized through a 0,22μm Sterivex-GS filter, aliquoted into 100ml sterile bottles and stored at 4°C. When required, the culture medium was saturated with CO_2 gas until the phenol red indicator turned yellow in colour. Complete medium was added to the parasite cultures, by addition of 10ml human plasma and 4,2ml of sterile 5% $NaHCO_3$ to 90ml incomplete medium prior to use. The $NaHCO_3$ was prepared by adding autoclaved water to 5g of $NaHCO_3$ up to a volume of 100ml which was then sterile filtered and stored at 4°C.

In certain drug sensitivity experiments, it was essential to use hypoxanthine-free medium. This medium was prepared as indicated above but with the omission of both hypoxanthine and gentamicin.

2.1.1.2 Plasma preparation

The plasma (AB^+) from at least 3 donors was pooled together and inactivated prior to use by heating at 56°C in a water bath for 2 hours. The inactivated plasma was then centrifuged at 800g (2700 rpm) for 10 minutes, and the supernatant aliquoted into sterile 10ml and 40ml tubes and stored at -20°C.

2.1.1.3 Erythrocyte preparation

According to Jensen and Trager (1976), at intervals of 3 or 4 days (or sooner, depending on the parasitaemia) fresh human erythrocytes (blood group O^+) are to be added to the malaria culture flasks. Human whole blood stored in citrate phosphate dextrose adenosine-1 was collected from a healthy donor, aliquoted out and stored at 4°C for a maximum period of three weeks. The blood was centrifuged for 5 minutes at 1500 rpm, the supernatant removed and the pellet resuspended in sterile PBS blood buffer (PBS buffer consists of 8,0g NaCl; 0,3g KCl; 0,73g Na_2HPO_2; 0,2g KH_2PO_4 in 1L water, which was sterilized

by being autoclaved at 120°C for 20 minutes under a pressure of 1,75 kgf/cm^2) . The blood was then washed twice (at 1500 rpm for 5 minutes) prior to addition the culture flasks to give a haematocrit of ±5%. The washed blood was resuspended in PBS to prevent dehydration, stored at 4 °C and used within 48 hours.

2.1.1.4 The culture smear and light microscopy: assessment of parasite growth

The percentage parasitaemia and parasite morphology was examined using the standard Giemsa staining procedure for thin smears.

The stain buffer was prepared by dissolving 3,5g of KH_2PO_2 and 14,55g of Na_2HPO_4 * 12 H_20 in distilled water, made up to a final volume of 1L. 2,5ml of Giemsa stain solution was required per slide, and neat Giemsa was dissolved in stain buffer at a ratio of 1:10. In the actual staining of the slide, the smear was air dried, fixed with 100% methanol and covered with Giemsa stain for 15 - 30 minutes. After rinsing the slide with water, it was examined under 100x oil immersion. The parasitaemia of the cultures was calculated according to the following formula:

$$\% \text{ PARASITAEMIA} = PC/(PC + EC) \times 100\ \%$$

Where: PC = Number of parasitised erythrocytes, and

EC = Number of uninfected erythrocytes

In order to use the above formula, 10 fields were studied. For each field, the number of infected and uninfected cells in ¼ of the field were counted and then multiplied by four.

2.1.2 SYNCHRONISATION OF CULTURES

Lambros and Vanderburg (1979), established the technique to synchronize erythrocytic stages of *P. falciparum* in culture prior to drug sensitivity testing. This technique is essential in the laboratory because *P. falciparum* grown *in vitro* loses the synchronicity it shows *in vivo*. Unless synchronization is carried out, it is impossible to harvest specific developmental stages of the parasite for experimental purposes. *Plasmodium falciparum* completes a life cycle every 48 hours. By using sorbitol to establish synchrony in the cultures, only young ring forms survive and grow. Older parasitic stages such as the trophozoites are selectively killed.

In order to establish synchrony, the culture was centrifuged at 1500rpm for 5 minutes, and the supernatant discarded. The pellet was resuspended in 10 volumes of 5% (w/v) D-sorbitol for 10-20 minutes at room temperature. After repeating the centrifugation step, the supernatant was removed and the pellet was transferred to a culture flask to which fresh, complete medium had been added. Cultures were re-established by adding uninfected erythrocytes, and fresh complete medium to the flasks.

2.1.3 FREEZING

In order to maintain frozen parasite stocks, it was essential to freeze cultures on a regular basis. The cultures had to be primarily in the early ring stage (parasitaemia > 5%), prior to freezing.

The culture was centrifuged at 1500rpm for 5 minutes and the supernatant removed. To the pellet , 28% glycerol in PBS was added in a 1:1 ratio. This suspension was left to stand at room temerature for 5 minutes. Approximately 0.5 ml of the parasite suspension was then aliquoted into sterile cryotubes, which were then stored in liquid nitrogen.

2.1.4 PREPARATION OF CULTURE FROM FROZEN STOCK (THAWING)

In order to re-establish an *in vitro* culture, the frozen cryotube was thawed in a 37°C waterbath and transferred into a calibrated centrifuge tube, 0. 1 ml of a 9% NaCl solution was added to each 1 ml of blood suspension, whilst shaking the tube. The tube was then left to stand at room temperture for 3-5 minutes. Nine volumes of sterile 1.6% NaCl was then added and mixed in gently. The tube was then centrifuged at 1500rpm for 5 minutes and the supernatant removed. The pellet was then gently resuspended in nine volumes of a 0.2% glucose and 0.9% NaCl solution and centifuged at 1500rpm for 5 minutes. After supernatant removal, the volume of the pellet was made up to 1ml with freshly washed blood and resuspended in complete culture medium. The suspension was placed in a culturing flask, incubated at 37°C and maintained as described in 2.1.1.

2.2 DRUG SENSITIVITY TESTING : THE HYPOXANTHINE UPTAKE METHOD OF ASSESSING *IN VITRO* GROWTH OF *P. FALCIPARUM*

2.2.1 THE BASIC HYPOXANTHINE METHOD

A semi-automated microdilution technique involving the incorporation of [^{3}H] hypoxanthine as an index of parasite viability, as developed by Desjardins *et al.*(1979) and illustrated in Figure 2.1, was used in subsequent experimentation. The activity of the immunopotentiating drug lentinan in combination with various monocyte culture supernatants, as well as the activity of curdlan sulphate alone and in combination with other classical antimalarials against cultured intra-erythrocytic asexual forms of the human malaria parasite *P. falciparum* was measured using this technique.

This method is "based on the inhibition of uptake of a radiolabelled nucleic acid precursor by the parasite during short term subculture in microtitration plates" (Desjardins *et al*, 1979, p 710). Liquid scintillation spectroscopy was used to quantify the amount of tritiated hypoxanthine incorporated into the parasite DNA during the experiment.

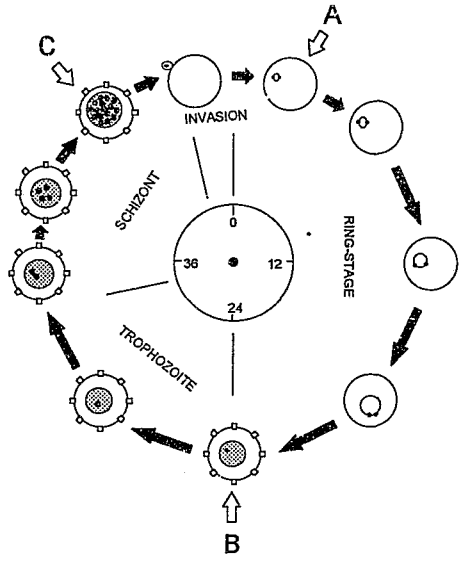

Figure 2.1 : The tritiated hypoxanthine assay in relation to the intraerythrocytic life cycle of the *P. falciparum* malaria parasite. (A) Drug addition; (B) ^{3} [H]-hypoxanthine addition; (C) Harvesting of parasite DNA.

2.2.2 PREPARATION OF DRUGS

All drug stock solutions were freshly prepared for each experiment and filter sterilized through a 0.22µm Millipore filter. Dilutions of the stock were carried out using hypoxanthine-free culture medium. The various stocks were prepared as follows:

2.2.2.1 Chloroquine

Chloroquine sulphate was dissolved in water.

2.2.2.2 Curdlan Sulphate

Curdlan sulphate was dissolved in phosphate buffered saline (PBS).

2.2.2.3 Lentinan

Lentinan was dissolved in 0.9 % NaCl.

2.2.2.4 Mefloquine

Mefloquine was dissolved in 10% ethanol in water. The ethanol concentrations never exceeded 1% of the final concentration, since this concentration has previously been shown to have no significant effect on the malaria parasite.

2.2.2.5 Quinine

Quinine HCl was dissolved in water.

2.2.3 ASSESSMENT OF PARASITE GROWTH

2.2.3.1 Preparation of uninfected control erythrocytes

Uninfected human erythrocytes were washed at least three times in PBS. They were then suspended at a 1% haematocrit in complete hypoxanthine-free medium.

2.2.3.2 Preparation of the parasites

Parasites were grown *in vitro* as described in section 2.1.1 and synchronised as in section 2.1.2. Prior to each microtitre plate experiment, cultures in the early ring stage (Figure 2.2) were diluted in hypoxanthine-free medium. Uninfected erythrocytes were added to yield a final hematocrit of 1% and a parasitaemia of 0,5%.

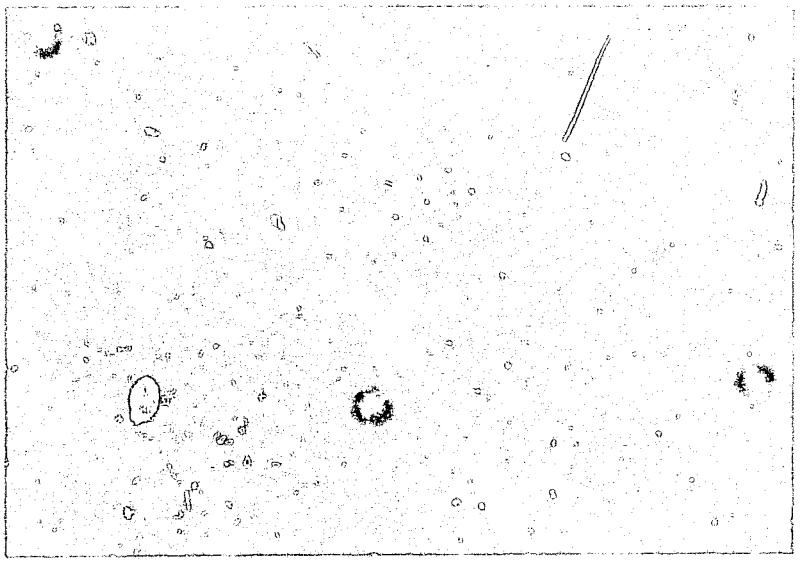

Figure 2.2: Smear of *P. falciparum* ring stages used in the hypoxanthine assay (1000x magnification)

2.2.3.3 Preparation of microtitration plates

Flat bottomed microtitre plates were used (Figure 2.3). Each plate contained 96 wells, arranged in a matrix of 8 rows (labelled A - H) and 12 columns (labelled 1 - 12).

- A single drug or a maximum of two drugs in combination were tested at one time. 25µl of the prepared drug dilution (or monocyte supernatant from various treatments), was added in triplicate to wells B1 - H12 of the plate. Thus, 28 different drug dilutions could be tested in each plate at a time.
- In order to maintain a fixed volume in each well, 25µl of hypoxanthine-free incomplete medium was pipetted into the EC (erythrocyte control - Row A, wells 1 to 4) and PC (parasite control - Row A, wells 5 to 12) wells.
- 200µl of a synchronized parasitized (early ring stage) erythrocyte suspension (section 2.2.3.2) was added to each well of the plate, except the EC wells. To the EC wells, 200µl of an uninfected erythrocyte suspension (section 2.2.3.1) was added.

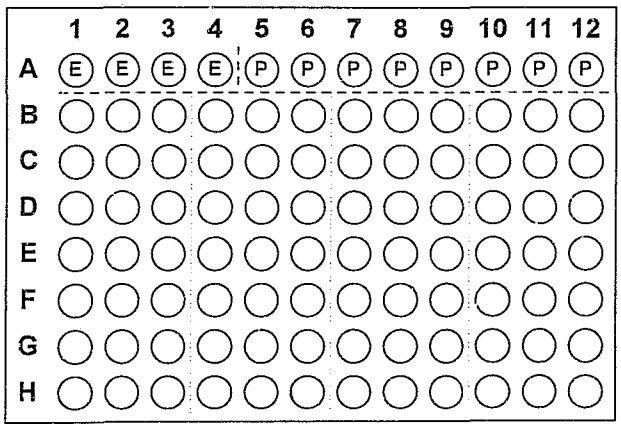

Figure 2.3: Schematic diagram of a 96-well microtitre plate for the ^{3}H-hypoxanthine assay. E is the uninfected erythrocyte control and P is the parasitised erythrocyte control.

- The plates were then incubated in a candle jar (section 2.2.3.4)

2.2.3.4 The Candle Jar Method

- The plates were placed into an airtight glass dessicator with a candle (Figure 2.4) The dessicator was swabbed down with 70% alcohol and left under UV light for 1 hour prior to use.
- The candle was lit and the cover secured but the stopcork was left open.
- When the candle was extinguished, the stopcork was replaced.
- The plates were incubated at 37°C for 24 hours.

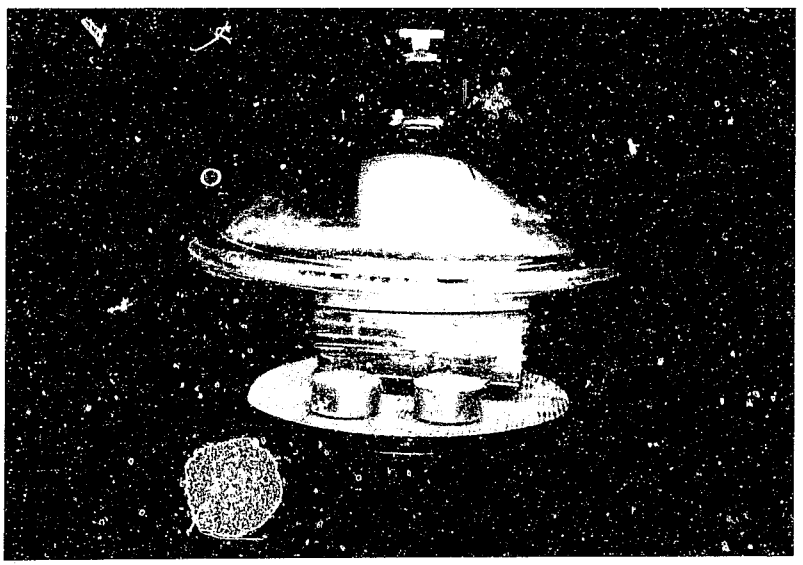

Figure 2.4: The candle jar used in hypoxanthine assays

2.2.3.5 Preparation of the isotope and labelling of parasites

As an index of parasite growth, the uptake of beta emitting [G-^{3}H]-hypoxanthine was measured. The hypoxanthine isotope was supplied in ampoules as a lyophylate. A stock solution was made by dissolving the contents of each ampoule in 2,0ml of 50% ethanol which was then stored at -20°C. Prior to being added to the microtitre plates, the ethanol was evaporated from 0.2 ml of the stock solution, leaving ± 0,1ml behind in the tube. 4,9ml of hypoxanthine-free complete medium was then added to the remaining ml of isotope. This 5ml was enough for two 96-well microtitration plates. 24 hours after the plates had been incubated, 25µl of the prepared isotope (at a concentration of 0,5µCi/well) was added to each well. The plates were then returned to the candle jar and incubated at 37^{0}C for a further 18 hours.

2.2.3.6 Harvesting and scintillation counting

After incubation for the required length of time, each plate was harvested using a semi-automatic cell harvester (Titertek Cell Harvester, Flow Laboratory). The instrument aspirated and deposited the particulate contents of each of the wells, which were washed three times with distilled water, onto small discs of glass fibre filter paper (Desjardins *et al.*,1979). Each disc (bearing the deoxyribonucleic acid of the parasites) then had to be dried and placed in a glass scintillation vial which contained 5ml of a toluene-based scintifluor (Aquagel) for counting. A liquid scintillation spectrometer was used to count all 96 vials (which in turn corresponded to the 96 wells on the microtitre plates). Each vial was counted for 1 minute.

2.2.3.7 Evaluation of disintegrations per minute (DPM) results

The percentage parasite survival was determined by calculating the mean DPM values for the erythrocyte controls, parasite controls and parasitised erythrocytes at each drug dilution which were then used in the

following formula:

$$\% \text{ Parasite survival} = (D - EC) \times (100/PEC)$$

Where: D = the mean dpm value of the parasitised erythrocytes at each drug dilution

EC = the mean dpm value of the uninfected erythrocyte control

PEC = the difference between the mean dpm values of the parasitized and uninfected erythrocyte controls.

2.2.3.8 Dose-response curves

Dose response curves were obtained for the different drugs using the computer program Enzfitter®. This program is a non-linear regression data analysis curve-fitting program based on Marquart's algorithm.

The following equation was used:

$$\% \text{ parasite survival} = 100/ (1 + (C/IC_{50})^P)$$

Where: C = the concentration of the drug

IC_{50} = 50% inhibitory concentration of the drug

p = the slope

From each dose response curve, an IC_{50} value was calculated. The IC_{50} value represents the molar concentration of a particular drug which results in a 50% decrease in [^{3}H]-hypoxanthine incorporation compared to the drug free controls i.e. the inhibitory concentration at which 50% of the parasites are killed.

2.2.4 MODIFICATIONS TO THE HYPOXANTHINE ASSAY FOR DOUBLE CYCLE COMBINATION DRUG EXPERIMENTS

In order to evaluate the effect of curdlan sulphate on re-invasion by merozoites, it was essential to carry out double cycle experiments over two schizogony cycles i.e a total incubation time of 78 hours with the following modifications:

- At T = 0 hours, 12.5 µl of the appropriately diluted classical antimalarial stock was added in triplicate to the wells of the microtitre plates
- 30 hours later (T = 30 hours), 12.5 µl of the appropriately diluted Curdlan Sulphate stock was then added to each of the wells in the same plates
- 30 hours later (T = 60 hours), 25µl of hypoxanthine was added to all the experimental wells
- 18 hours later (T = 78 hours), the plates were harvested

2.3 MONOCYTE SEPARATION AND CULTURING *IN VITRO*

2.3.1 FICOLL-HYPAQUE SEPARATION OF MONONUCLEAR CELLS FROM WHOLE PERIPHERAL BLOOD

2.3.1.1 Principle:

According to Miller *et al.*(1991), the separation of mononuclear cells from whole blood involves overlaying whole peripheral blood onto a lymphocyte separation medium (LSM) such as Ficoll-Hypaque which has a specific gravity of 76 to 80. After centrifugation, 5 distinct layers will be observed: from top to bottom these are plasma and saline, mononuclear cells and platelets, LSM, granulocytes and

erythrocytes (Figure 2.5). The recovery of lymphocytes and monocytes is achieved by initially harvesting, and then washing the mononuclear cell band.

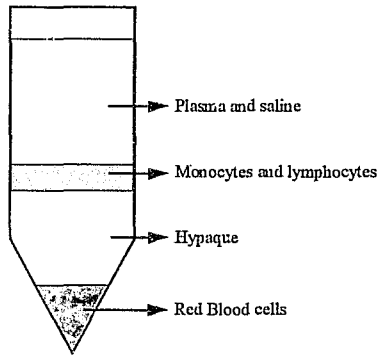

Figure 2.5: Representation of the different layers observed after centrifugation of blood on Ficoll-Hypaque

2.3.1.2 Specimen collection and preparation

5-10ml of heparinized blood, the usual specimen requirement (the minimum volume of blood used would depend on the lymphocyte count of the patient), was used. Anticoagulants other than heparin may also be used (e.g. 0,38% sodium citrate). The blood was kept at room temperature and used within 4 hours of collection.

2.3.1.3 The separation procedure was carried out according to a modification of the method proposed by Boyum (1968)

1) Blood was collected in 3 x 10ml heparinised blood tubes

2) Blood was spun for 10 minutes at 1500 rpm (Figure 2.6)

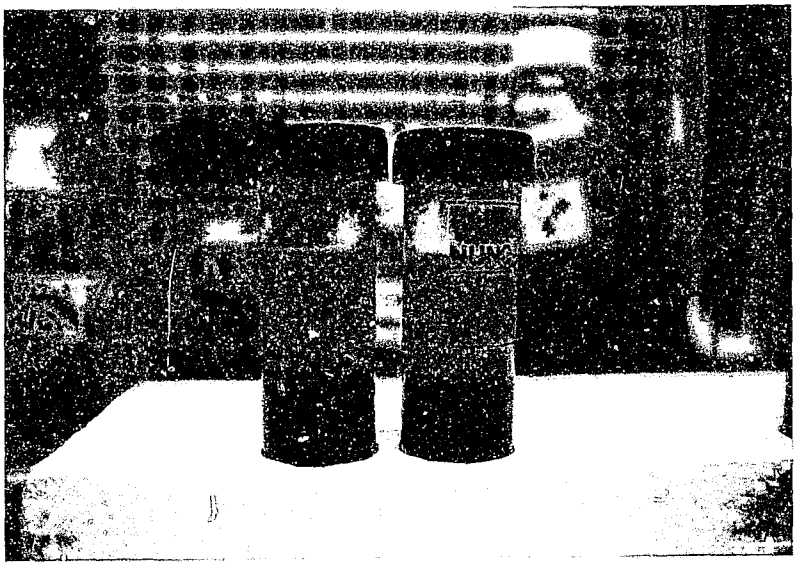

Figure 2.6: The appearance of whole peripheral blood after centrifugation at 1500rpm for 10 minutes

3) Plasma was removed to about 2-3 ml of the buffy coat

4) The buffy coats were retrieved (~ 1,5 - 2 ml using a pipette) and diluted up to 30 ml with saline.

5) This mixture was added to 15 ml Ficoll-Hypaque gradient (Figure 2.7). The sample was added slowly down the side of the tube without disturbing the Ficoll-Hypaque. The more care taken to layer the blood initially, the more distinct the mononuclear band was after centrifugation.

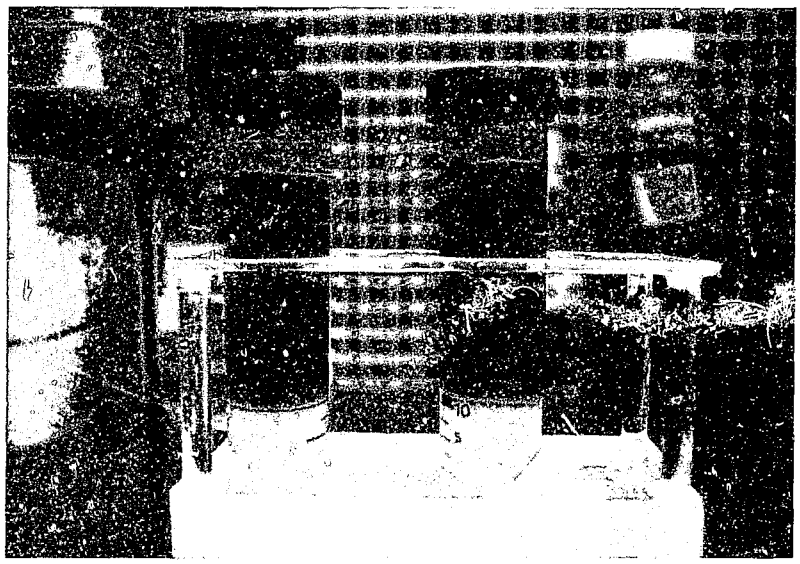

Figure 2.7 : The appearance of diluted blood layered onto Ficoll-Hypaque gradient

6) The mixture was spun at 2 400 rpm for 15 minutes

7) Mononuclear cells were at the plasma/saline interface (Figure 2.8)

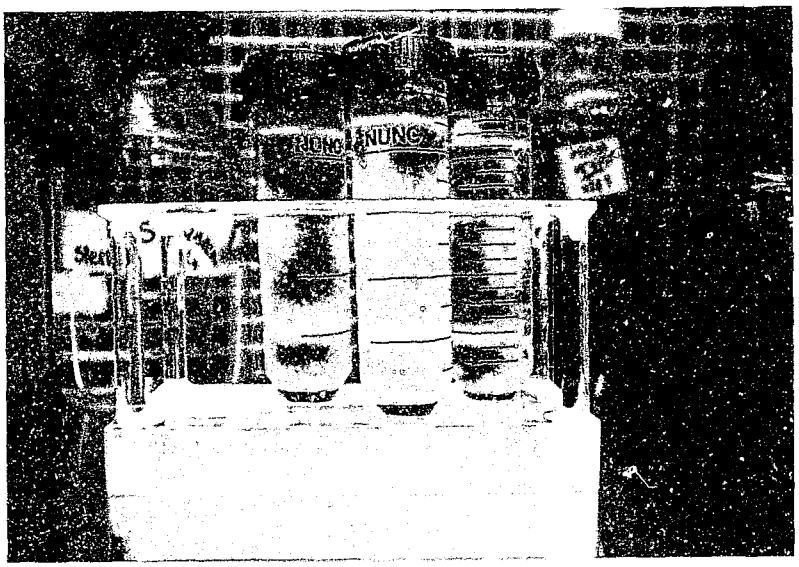

Figure 2.8: The mononuclear cell band which appears after centrifugation

9) Plasma and saline were removed to within about 1 ml of the buffy coat

10) 2 buffy coats were placed into one 50 ml centrifuge tube and diluted with saline up to 50ml

12) The tubes were spun at 1 200 rpm for 10 mins

13) Medium and plasma were at the top, and the mononuclear cells at the bottom

14) The plasma and medium were removed (poured off) and the pellet remained at bottom

15) The pellet was resuspened in 2% RPMI (up to 12 ml) in each 50 ml tube and mixed, but not vortexed

2.3.1.4 Separation of monocytes from lymphocytes (Goode et al, 1991)

Monocytes are adherent cells (i.e. stick to glass and plastic), while lymphocytes are relatively non-adherent. This difference was used to obtain a crude separation of the two cell types.

The cells were plated into glass petri dishes (Figure 2.9) .

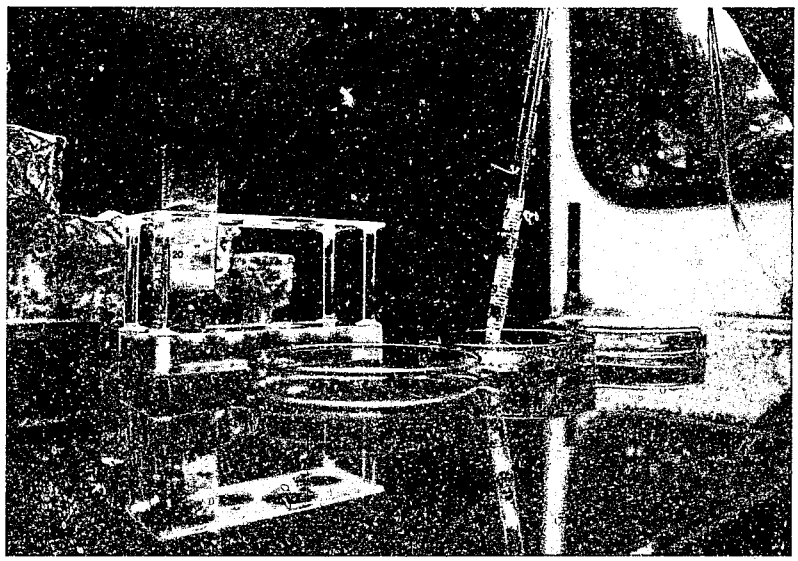

Figure 2.9: Plating out of monocytes into large glass petri dishes

The dishes were then incubated for 90 mins - 2 hours at 37°C (95% air, 5% $C0_2$), for cells to adhere. The supernatant was poured off from the plates . The plates were washed once with RPMI in order to remove non-adherent lymphocytes (T and B cells). A culture of pure monocytes then remained.

2.3.1.5 Trypsin/EDTA treatment for removal of monocytes from plates

A trypsin/EDTA mixture, poured so that it just covered the bottom of the plates, was left for 3-5 minutes. The mixture was pipetted into a centrifuge tube and spun for 5 minutes at 1200 rpm. The supernatant was poured off and the cells were washed once with RPMI. They were finally resuspended in complete monocyte culture medium (refer to 2.3.3)

2.3.2 COUNTING OF MONOCYTES

The cells were stained with Trypan blue and cell concentration was determined using a haemocytometer.

2.3.2.1 Staining of the monocytes

The Trypan blue was diluted in a 1:4 ratio (0,25 µl Trypan to 0,75 µl PBS), and then a 25 µl sample of the monocytes was mixed with 75 µl of the diluted dye.

2.3.2.2 Using the haemocytometer to determine monocyte counts:

- With the cover-slip in place, a Pasteur pipette was used to transfer a small volume of the cell suspension

to both chambers of the haemocytomer (the edge of the cover-slip was touched carefully with the pipette tip and each chamber was allowed to fill by capillary action). The chambers were neither overfilled nor underfilled .

- Starting with 1 chamber of the haemocytometer, all the cells in the 1mm centre square and four 1mm corner squares were counted

Note: The cells on the top and left touching middle line at the bottom and right sides were counted.

-This procedure was repeated for chamber 2.

- **Cell Counts** - Each square of the haemocytometer, with cover-slip in place, represents a total volume of mm^3 or $10^{-4}cm^3$. Since $1cm^3$ is equivalent to 1ml, the subsequent cell concentration per ml (and the total number of cells) was determined using the following calculations:

CELLS PER ml = the average count per square mm x dilution factor x 10^4

TOTAL CELLS = number of cells per ml x the original volume of fluid from which cell sample was removed

- A second sample was withdrawn and the counting procedure repeated to ensure precision.

2.3.3 CULTURING OF THE MONOCYTES

The purified monocytes were re-adhered to the glass plates and cultured in liquid suspension.

The monocyte culture medium was prepared as follows (Danis *et al.*, (1991) :

- RPMI 1640 (10,4g/L) buffered with 20 mM HEPES (4,77g/L) and 10mM $NaHCO_3$ (2g/L) (pH - 7.4)

  The medium was supplemented with:
- 2mM L-glutamine (1ml/100ml medium to be added every 4 weeks)
- 5-10% Heat-inactivated Fetal Calf Serum (10ml/100ml medium)
- Gentamycin (100µl/100ml medium)

Cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5%.

2.4 *IN VIVO* MODEL (Peters *et al.*, 1987)

2.4.1 INFECTION OF ANIMALS

2.4.1.1 Animal care

Inbred female Balb/c mice, 6-8 weeks old and weighing 20-25g were housed in a temperature controlled room (22°C) with a reversed light:dark cycle of 12:12 hours. The mice were fed chow and supplied tap water *ad libitum*.

2.4.1.2 Parasites

A chloroquine sensitive strain of *Plasmodium berghei* obtained from the SAIMR was used. The parasites were stored in liquid nitrogen at a ratio of 1:2 of infected blood and 28% glycerol.

2.4.1.3 Development of experimental inoculum

Generally, 6 mice were used for the development of experimental inoculum. Initially, 2 mice were inoculated intraperitoneally with 0.1 ml *P.berghei* after the frozen vial had been thawed. The percentage parsitaemia was determined daily (see section 2.4.2.1). When the parasitaemia approached ± 50%, these mice became donors. The mice were anaesthestised by intramuscular injection of Innovar-Vet (0.4 mg fentanyl and 20 mg/ml droperidol, Janssen AH), at 0.2 mg/kg of body weight. Once anaesthetised, a mid-line abdominal incision was made and the organs were moved to one side to facilitate location of the caudal vena cava. The caudal vein was punctured using a 22-guage hypodermic needle and blood was drawn into a syringe into which 1ml EDTA had been placed to prevent coagulation of the blood. The collected blood was then transferred into a tube with 1ml

sterile phoshate-buffered saline. The animals were then euthanased by injecting ml of Euthanase into the heart. Another 4 recipient mice were immediately infected with ml of the inoculum collected from the 2 donor mice above. When the parasitaemia approached 50%, the blood was collected as described and transferred from the syringe to an EDTA blood tube. The blood was pooled and centrifuged at 2000rpm for 2 minutes. The pelleted cells were then diluted with sterile PBS to obtain a concentration of 10^7 parasitised erythrocytes/ml (PRBC's). This was the experimental inoculum used to infect the mice in the main experimental groups.

2.4.1.4 Infection of experimental animals

Experimental animals were infected intraperitoneally with 0.1 ml *P.berghei* inoculum, containing 10^6 parasitised erythrocytes unless otherwise indicated. The course of infection was then monitored as described above.

2.4.1.5 Haematocrit determination in experimental animals

On the "end day" of experimentation (when either (i) the body mass had dropped by 15% or (ii) the parasitaemia had reached ~ 50%), the animals were anaesthetised and bled from the tail vein in order to obtain blood for haematocrit calculation. This was technically easier than obtaining blood from the abdominal aorta or intracardially, and had been approved by the Central Animal Services Unit (CASU). The blood was collected from the tail tips in heparinised capillary tubes and immediately centrifuged at 10000g for 5 min at room temperature (Brown, 1988).

2.4.1.6 Calculation of Spleen Indices in experimental animals

Subsequent to obtaining blood for haematocrit determination, the animals were euthanased and their spleens surgically removed and weighed in order to calculate spleen indices according to the following formula (Forget, 1981) :

$$\text{SPLEEN INDEX} = \sqrt{\frac{\text{Spleen weight x } 100}{\text{Body weight}}}$$

2.4.2 MONITORING OF CLINICAL COURSE OF INFECTION

The parasitaemia, weight and other clinical signs were monitored in all animals i.e those used for the development of experimental inoculum as well as those used in drug testing experiments.

2.4.2.1 Determination of parasitaemia

Percentage parasitaemia was determined for each animal every 24 hours by taking a drop of blood from the tail, and preparing a smear for microscopic analysis. The blood film was fixed with methanol and stained for 30 minutes in 5% (v/v) solution of Giemsa stain. On each slide, the number of parasitised cells in 5 fields were counted under oil immersion (1000x) and the data was expressed as the percentage of parasitised erythrocytes.

2.4.2.2 Weight

The mice were weighed daily. The animals were euthenased if their total body weight dropped by more than 15%.

2.4.2.3 Clinical signs of infection

Clinical signs such as: activity, blood perfusion of ears, colour of the urine and condition of the fur were also monitored daily.

3. CHAPTER THREE - *IN VITRO* EXPERIMENTATION WITH LENTINAN

3.1.1 INTRODUCTION

Polysaccharides with antitumour activity have been isolated from diverse sources including higher plants, fungi, lichens, yeasts and bacteria (Chihara, *et al.*, 1969).

The polysaccharide lentinan, is a ß1,3-glucan which is reported to have immunomodulating characteristics. In addition to increasing host resistance against human and murine tumours, lentinan has been found to augment immune responses to a variety of antigens including bacteria, viruses and parasites (Diluzio *et al.*, 1981). Its immunological effects are mediated through both mononuclear cells and T-cells, triggering the production of various cytokines associated with immunity (Maeda *et al.*, 1973) for example, by stimulating murine macrophages to produce IL-1 (Hamuro, 1980).

Lentinan is also reported to be effective in boosting host resistance against parasitic schistosomal infections (Byram *et al.*, 1979), and yeast glucans are reported to effectively boost the immune system of certain animals infected with *Plasmodium berghei* or *Leishmania donovei* (Song and Diluzio, 1979). To date however, the effect of lentinan on host resistance against *Plasmodia* has not been determined. This gap in research with lentinan, highlighted the need to carry out an investigation on the effectiveness of lentinan against *P. falciparum.*

3.2 AIMS

* To activate the monocytes by stimulation with lentinan and other culture components
* To investigate the effect of monocyte products on *P. falciparum* growth *in vitro*
* To determine the effect of products from monocytes stimulated *in vivo* on *P. falciparum* growth *in vitro*

3.3 METHODOLOGY

3.3.1 THE DIRECT EFFECT OF LENTINAN ON *P. FALCIPARUM*

3.3.1.1 Preparation of Lentinan stock solution and dilutions

A 2mg/ml solution was made up according to section 2.2.2.3. Drug concentrations ranging from 1µg - 500µg/ml were then prepared and diluted 10 x in the 96 well microtitre plates.

3.3.1.2 The hypoxanthine assay

Each concentration was tested in repeats of 4 in the hypoxanthine uptake method (section 2.2). This experiment was repeated 3 times.

3.3.2 THE EFFECT ON *P. FALCIPARUM* OF 24 HOUR LENTINAN STIMULATED MONOCYTE SUPERNATANTS

These experiments were carried out in order to determine the optimum concentration combinations of monocytes and lentinan effective against *P. falciparum.*

3.3.2.1 Prepration of the monocyte and lentinan combinations

(i) Monocytes were separated according to method 2.3.1.

(ii) The monocytes were suspended in 4 tubes (A, B, C, D) in 1ml of monocyte culture medium and counted using a haemocytometer. Monocyte counts were ~ 10^6 cells/ml in each tube.

(iii)
- 100µl of cells from Tube A were plated into each of four small plastic Nunclon plates (L_1A, L_2A, L_3A, L_4A)
- 100µl of cells from Tube B were plated into each of four small plastic Nunclon plates (L_1B, L_2B, L_3B, L_4B)
- 100µl of cells from Tube C were plated into each of four small plastic Nunclon plates (L_1C, L_2C, L_3C, L_4C)
- 100µl of cells from Tube D were plated into each of four small plastic Nunclon plates (L_1D, L_2D, L_3D, L_4D)

(iv) A 1000µg/ml lentinan stock solution was prepared according to section 2.2.2.3.

The following dilutions of the stock were then made: 750µg/ml, 500µg/ml, 250µg/ml, 100µg/ml.

(v) 1ml of each stock solution was plated out as follows:

PLATES	STOCK SOLUTION
L_1A, L_1B L_1C, L_1D	750 µg/ml
L_2A, L_2B L_2C, L_2D	500 µg/ml
L_3A, L_3B L_3C, L_3D	250 µg/ml
L_4A, L_4B L_4C, L_4D	100 µg/ml

Since varying volumes of monocytes were already present in the plates on addition of the 1ml of lentinan, the adjusted concentrations of monocytes as well as the adjusted concentrations of lentinan, were re-calculated.

(vi) The monocytes were left in culture with the lentinan for 24 hours.

(vii) The supernatants were removed from the plates and spun down at 1 500 rpm for 10 mins to remove cellular debris.

(vii) The supernatants were stored in Eppendorfs at -20° C until further use in the hypoxanthine assays.

3.3.2.2 Hypoxanthine Assay

A hypoxanthine assay was carried out according to section 2.2 and repeated for verification of results. Each monocyte and lentinan combination (e.g. L_1A, L_2A....) was tested in repeats of 5.

3.3.3 THE EFFECT ON *P. FALCIPARUM* OF 12 HOUR AND 12 HOUR HEAT INACTIVATED SUPERNATANTS FROM LENTINAN STIMULATED MONOCYTES

These experiments were carried out in order to determine the effect on *P. falciparum* of supernatants from various combinations of monocyte and lentinan concentrations. The monocytes were stimulated with the Lentinan for 12 hours and then the effect of the 12 hour supernatants as well as 12 hour heat-inactivated supernatants were tested on the parasites.

3.3.3.1 Preparation of the monocyte and lentinan combinations

This experiment was carried out in accordance with section 3.3.2 above, but the following changes were made:

(i) The monocytes were left in culture with the lentinan for 12 hours.

(ii) Half of the supernatant samples (all combinations) were stored at -20°C. The other half were heat-inactivated at 56°C for 30 minutes. After inactivation, the supernatants were stored at -20°C.

3.3.3.2 Hypoxanthine assay

A hypoxanthine assay was carried out according to 2.2 and repeated for verification of results.

Each combination (non heat-inactivated and heat-inactivated) was tested in repeats of 7.

3.3.4 PILOT STUDY : THE EFFECT ON *P. FALCIPARUM* OF 24 HOUR SUPERNATANTS FROM VARIOUS MONOCYTE TREATMENTS

In this pilot experiment, the effect on *P. falciparum* of supernatants from monocytes stimulated for 24 hours with combinations of lentinan, parasite culture medium and parasites directly was investigated.

3.3.4.1 *Monocyte isolation and treatments*

- Monocytes were isolated from whole peripheral blood according to section 2.3.1 and counted using a haemocytometer.

  The cells were then separated to give :

A - 10^5 monocytes/ml, plated out into each of 6 petri dishes

B - 10^4 monocytes/ml, plated out into each of 6 petri dishes.

- The monocytes in each of the 12 petri dishes above were then stimulated with either lentinan, parasite culture medium (supernantant) and parasites according to the following layout:

(i) **A - 10^5 cells + 10 µg/ml lentinan**

Treatment in each plate:

A1.	Control	→	monocytes in RPMI
A2.	Lentinan	→	monocytes stimulated with 10 µg/ml lentinan
A3.	Culture medium (supernatant)	→	monocytes stimulated with parasite culture medium
A4.	Parasites	→	monocytes stimulated with parasites directly (parasitaemia of 3%)
A5.	Lentinan + culture medium culture	→	monocytes stimulated with 10 µg/ml lentinan and parasite medium (supernatant) simultaneously.
A6.	Lentinan + parasites	→	monocytes stimulated with 10 µg/ml lentinan and parasites simultaneously.

(ii) **B - 10^4 cells + 20 µg/ml lentinan**

Treatments in these 6 petri dishes (B 1 -6) were in accordance with (i) above, except that the monocytes were stimulated with 20 µg/ml lentinan instead of 10 µg/ml lentinan.

- The monocytes were left in culture for 24 hours.
- The supernatants from each of the 12 plates (A1-6, B1-6) were harvested and centrifuged at 1500 rpm for 10 mins to remove any cellular debris.
- The supernatants were stored at -20°C until further use in the hypoxanthine assay.

3.3.4.2 Hypoxanthine Assay

- A hypoxanthine assay was carried out according to section 2.2.1 and repeated for verification of results.

- Each treatment (A1-6, B1-6) was tested in repeats of 14.

3.3.5 THE EFFECT ON *P. FALCIPARUM* OF 12 HOUR AND 12 HOUR HEAT INACTIVATED SUPERNATANTS FROM VARIOUS MONOCYTE TREATMENTS

In these experiments, the effect on *P. falciparum* of supernatants from monocytes stimulated for 12 hours with combinations of lentinan, parasite culture medium and parasites directly was investigated. The effect of heat-inactivating the 12 hour supernatants was also investigated.

3.3.5.1 Experimental procedure

- This experiment was carried out in accordance with section 3.3.3, with the following changes :

1) Stimulation of the monocytes for 12 instead of 24 hours.

2) In an effort to determine the nature of the active components in the supernatants, the supernatants were tested both directly and after heat-inactivation at 56°C for 30 minutes.

3.3.5.2 Hypoxanthine Assay

A hypoxanthine assay was carried out according to section 2.2 and repeated for verification of results. Each treatment A1-6, B1-6, A1-6(heat inactivated) and B1-6 (heat inactivated) was tested in repeats of 7.

3.4 RESULTS

3.4.1 THE DIRECT EFFECT OF LENTINAN ON *P. FALCIPARUM*

The effect of Lentinan at varying concentrations (from 0.1 to 60µg/ml) on *P. falciparum* survival *in vitro* is shown in Figure 3.1 below. Lentinan was found to exert no direct effect on *P. falciparum in vitro*. This experiment was performed in triplicate, with the same results each time.

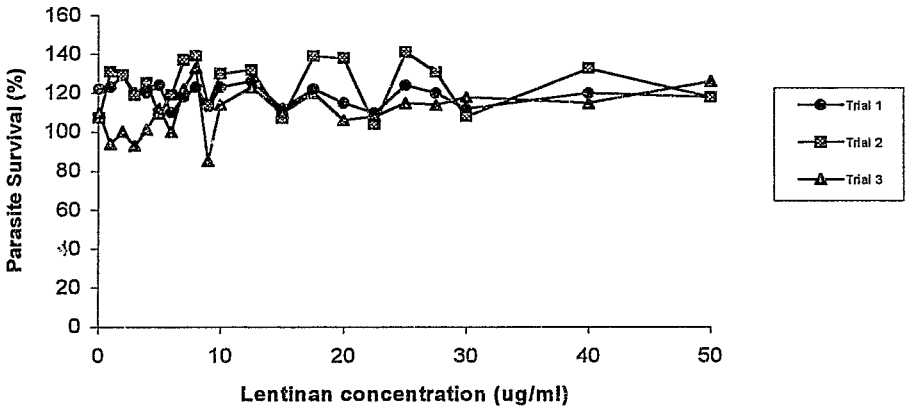

Figure 3.1: The direct effect of lentinan on *Plasmodium falciparum* survival *in vitro*. The experiment was carried out in triplicate.

3.4.2 THE EFFECT ON *P. FALCIPARUM* OF 24 HOUR, 12 HOUR AND 12 HOUR HEAT INACTIVATED SUPERNATANTS FROM COMBINATIONS OF MONOCYTE AND LENTINAN CONCENTRATIONS

The most effective Lentinan and monocyte combination for maximum inhibition of parasite survival (survival ~ 25%) after 24 hour stimulation of the monocytes appears to be 10^5 cells stimulated with 10µg/ml Lentinan (Figure 3.2).

Stimulation of the various concentrations of monocytes with various concentrations of lentinan for 12 and 24 hours (Figures 3.2 & 3.3), did not reduce parasitaemia. There was no monocyte/lentinan combination effective against *Plasmodium falciparum*. Although percent parasite survival increased by ~ 25% after heat inactivation of the 12 hour supernatants (Figure 3.4), the monocyte/lentinan combinations were still ineffective in reducing parasitaemia.

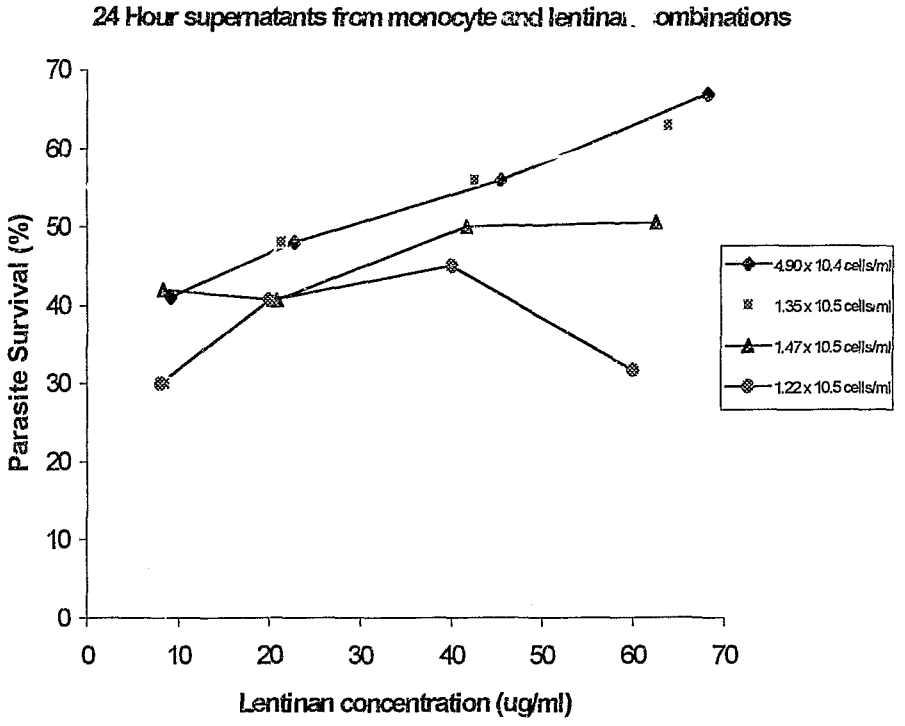

Figure 3.2: The effect of 24 hour supernatants from combinations of monocyte and lentinan concentrations on the survival of *P. falciparum* .

* Monocyte counts given as cells/ml.

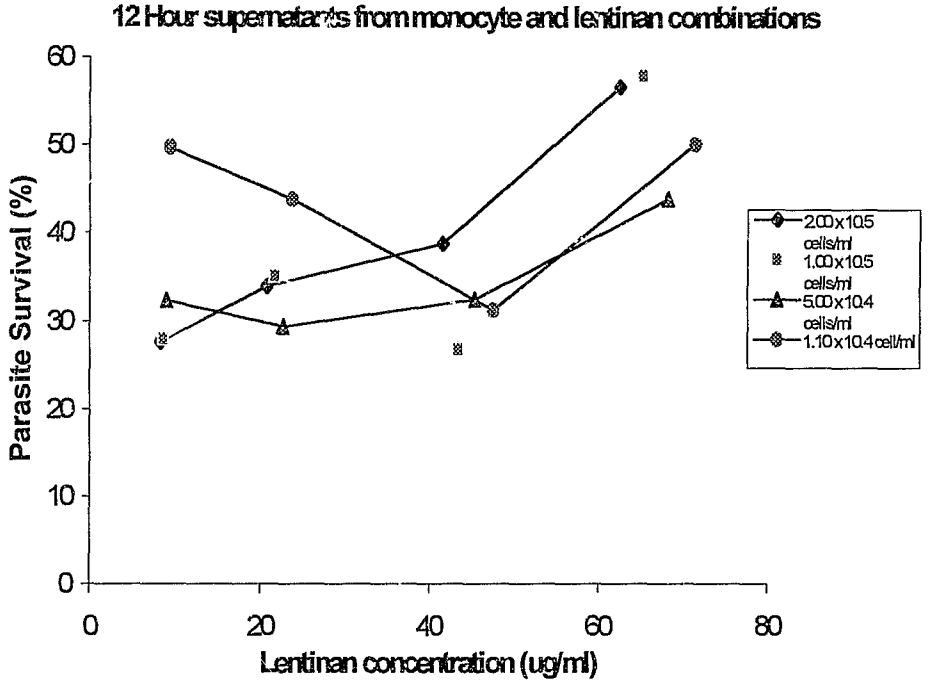

Figure 3.3: The effect of 12 hour supernatants from combinations of monocyte and lentinan concentrations on the survival of *P. falciparum.*

* Monocyte counts given as cells/ml

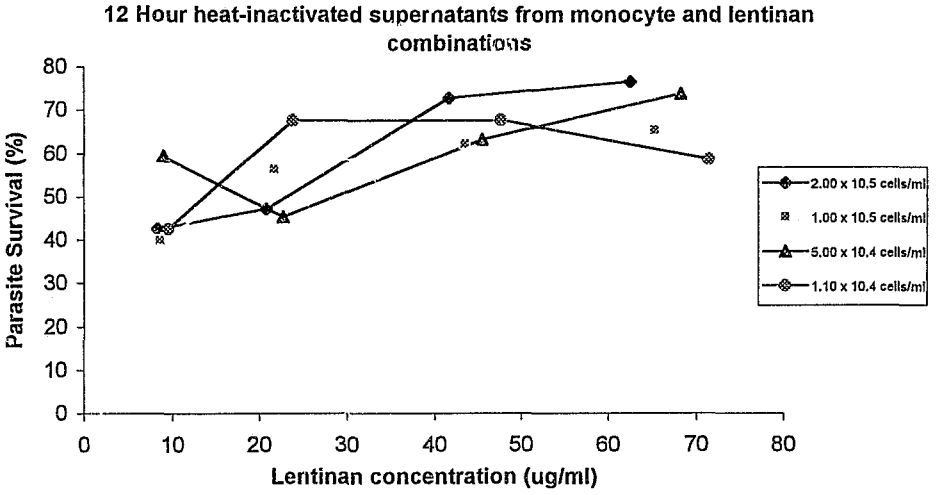

Figure 3.4: The effect of 12 hour heat inactivated supernatants from combinations of monocyte and lentinan concentrations on the survival of *P. falciparum.*

* Monocyte counts given as cells/ml

3.4.3 THE EFFECT ON *P. FALCIPARUM* OF 24 HOUR, 12 HOUR AND 12 HOUR HEAT-INACTIVATED SUPERNATANTS FROM VARIOUS MONOCYTE TREATMENTS

THE MONOCYTE TREATMENTS WERE AS FOLLOWS: THE MONOCYTES WERE STIMULATED WITH COMBINATIONS OF LENTINAN, PARASITE CULTURE MEDIUM AND PARASITES DIRECTLY

With reference to Figure 3.5, when 10^5 cells/ml were stimulated with 10μg/ml lentinan, maximum inhibition of parasite survival occurred when supernatants were used from monocytes stimulated for 24 hours with parasite culture medium. The percent parasite survival was decreased by a lesser extent when the monocytes were stimulated directly with the parasites, and the least inhibition of parasite growth was observed when the monocytes were unstimulated. In all three cases, when lentinan was added to the monocyte culture, the parasitaemia increased slightly.

When 10^4 cells/ml and 20μg/ml lentinan were used (Figure 3.6), similar results were obtained for the various treatment groups but the percent parasite survival increased between 5 - 10%. In comparison to the 24 hour stimulation, the 12 hour stimulation of 10^5 cells with 10μg/ml lentinan (Figure 3.7), resulted in a decrease in percent parasite survival of between 5-10% in certain treatment groups - M, M+L, M+C, M+L+C, C+L. Heat inactivation of these 12 hour supernatants (Figure 3.8), resulted in an increase of 5 - 10% in parasite survival in all treatment groups.

Compared to the 24 hour stimulation, the 12 hour stimulation of 10^4 cells/ml with 20μg/ml lentinan (Figure 3.9) decreased the percent parasite survival in treatment groups M, M+L, M+C by 3 - 15%. Heat inactivation of these 12 hour supernatants (Figure3.10) resulted in an increase in survival of between 5-10% in all treatment groups.

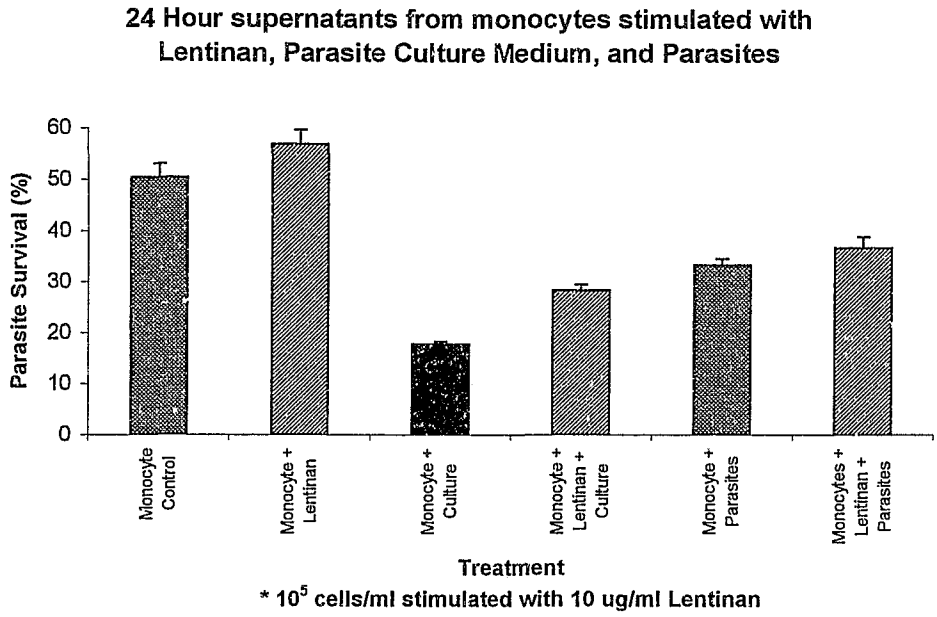

Figure 3.5: The effect on *Plasmodium falciparum* of 24 hour supernatants from monocytes stimulated with combinations of lentinan (L), parasite culture medium (c) and parasites (p).

* 10^5 cells/ml stimulated with 10μg/ml

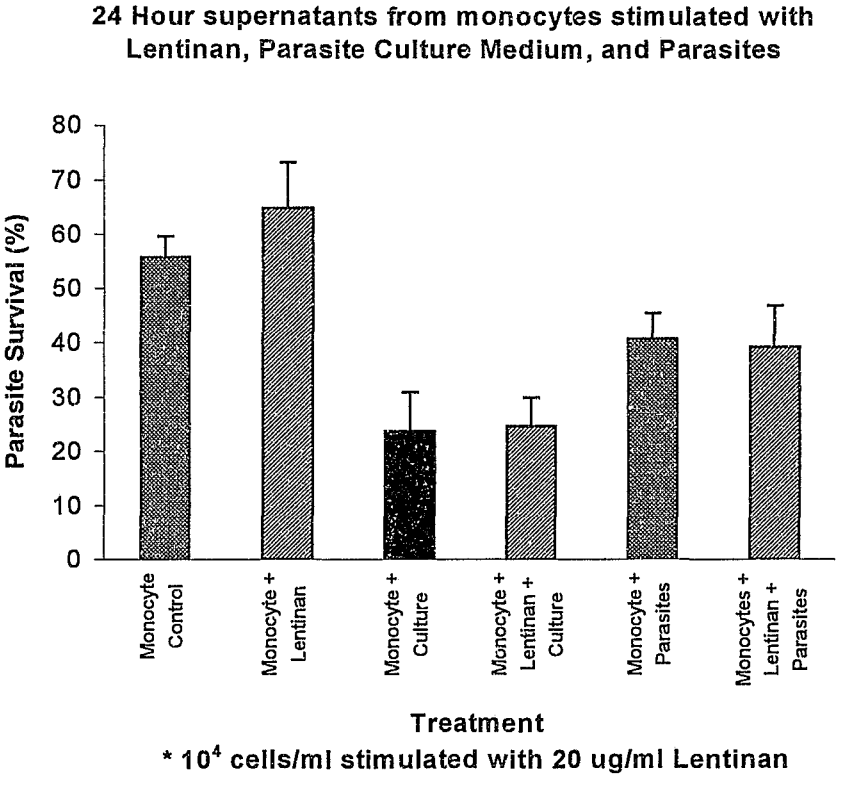

Figure 3.6: The effect on *Plasmodium falciparum* of 24 hour supernatants from monocytes stimulated with combinations of lentinan (L), parasite culture medium (c) and parasites (p).

* 10^4 cells/ml stimulated with 20μg/ml lentinan

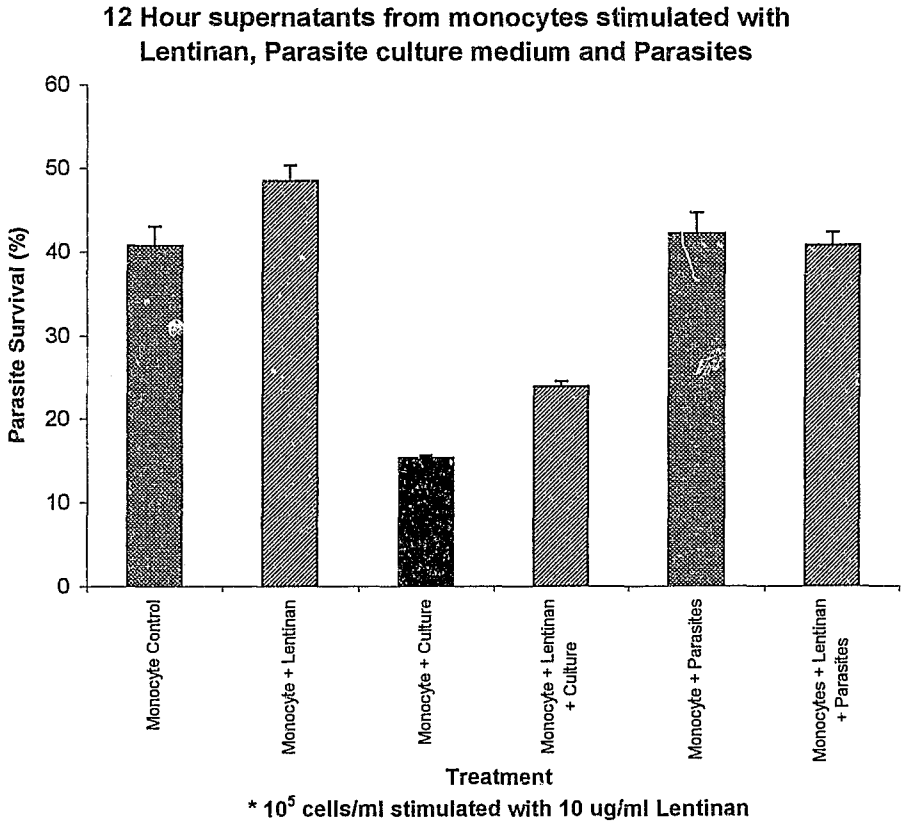

Figure 3.7: The effect on *Plasmodium falciparum* of 12 hour supernatants from monocytes stimulated with combinations of lentinan (L), parasite culture medium (c) and parasites (p).

* 10^5 cells/ml stimulated with 10μg/ml lentinan

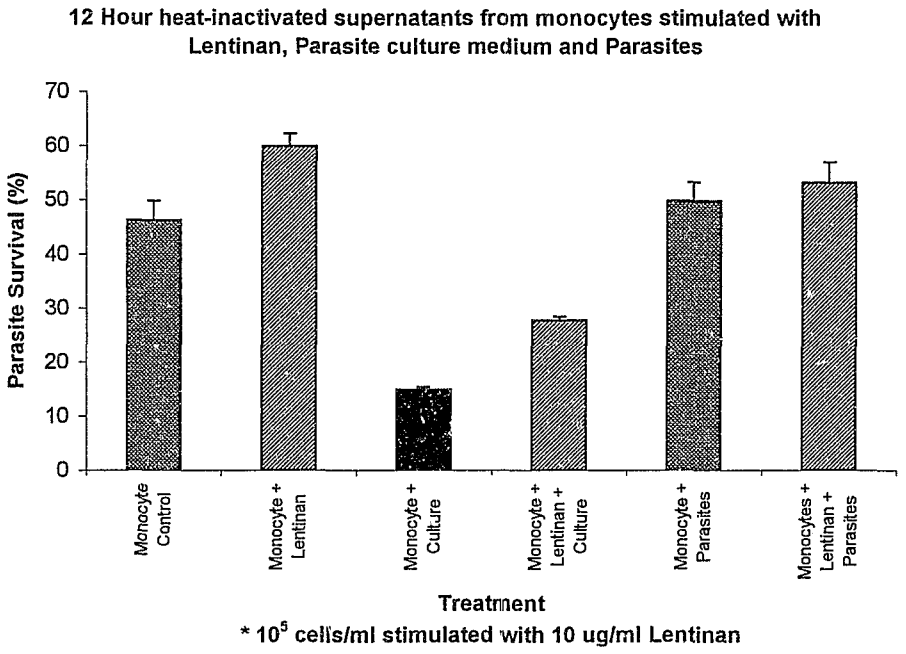

Figure 3.8: The effect on *Plasmodium falciparum* of 12 hour heat inactivated supernatants from monocytes stimulated with combinations of lentinan (L), parasite culture medium (c) and parasites (p).

* 10^5 cells/ml stimulated with 10μg/ml lentinan

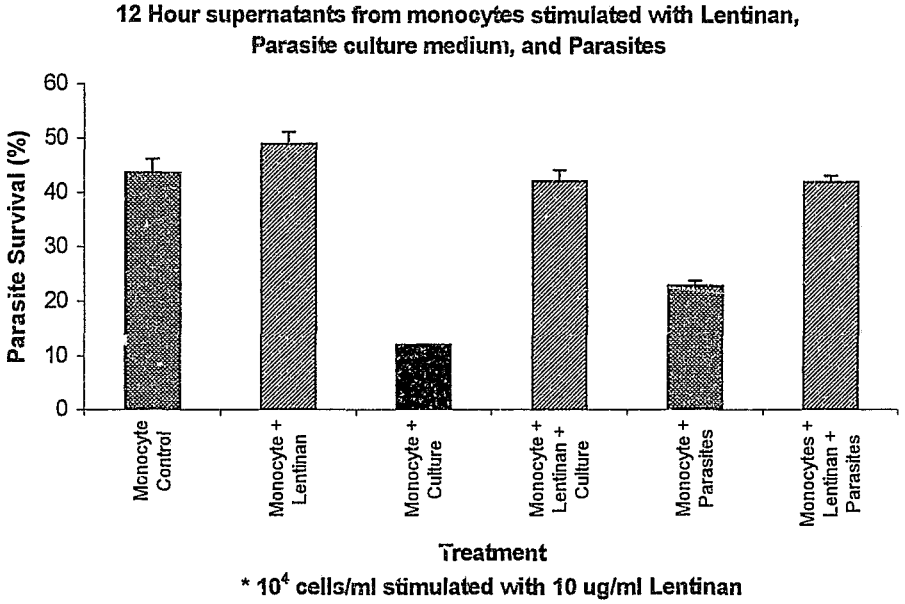

Figure 3.9: The effect on *Plasmodium falciparum* of 12 hour supernatants from monocytes stimulated with combinations of lentinan (L), parasite culture medium (c) and parasites (p).

* 10^4 cells/ml stimulated with 20μg/ml lentinan

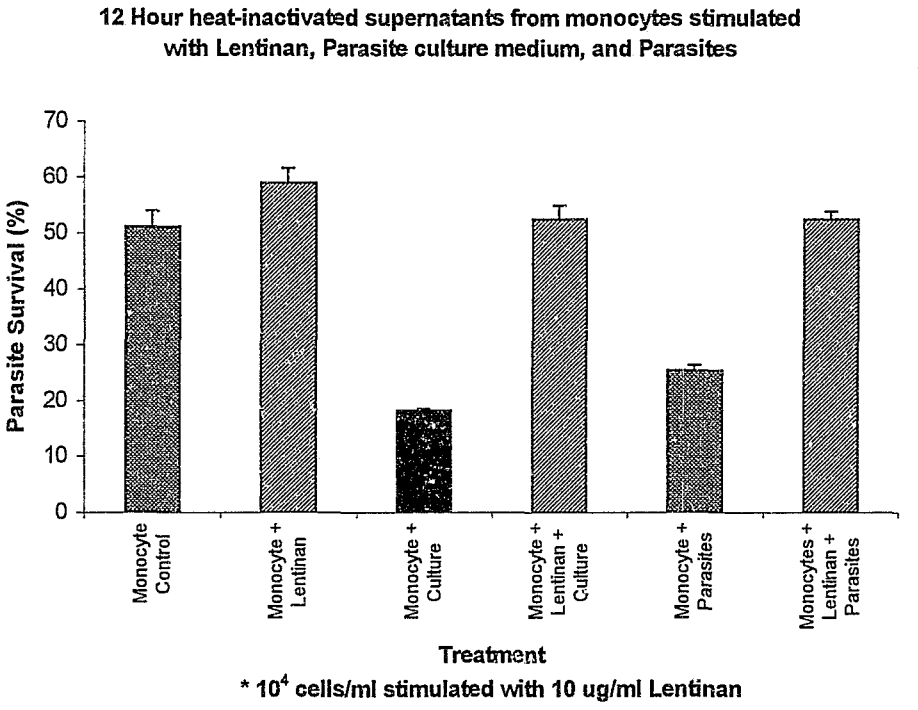

Figure 3.10: The effect on *Plasmodium falciparum* of 12 hour heat inactivated supernatants from monocytes stimulated with combinations of lentinan (L), parasite culture medium (c) and parasites (p).

* 10^4 cells/ml stimulated with 20μg/ml lentinan

3.5 DISCUSSION

Initial *in vitro* investigation involved the determination of whether or not lentinan had a direct effect on parasite survival, as well as the determination of the most effective lentinan and monocyte concentration combinations effective against the parasite.

Lentinan was found to exert no direct effect on *P. falciparum in vitro* (Figure 3.1). Since lentinan is an immuno-modulating agent and hence requires the cells of the immune system in order to exert its effect, this result was expected.

The effect of varying concentrations of monocytes stimulated with varying concentrations of lentinan, on *P. falciparum,* was then assessed. The results indicate an inconclusive effect of the lentinan and monocyte combinations on *P. falciparum* (Figs 3.2 & 3.3). After heat inactivation of the 12 hour supernatants (Figure 3.4), the percent parasite survival increased by ~ 25%. Since cytokines are glyco- or poly-peptide molecules, this increase can possibly be explained by a denaturation of the protein structure of the molecules, with a subsequent decrease in cytokine effectiveness .

Subsequent *in vitro* experimentation, involved the determination of the difference in effect on *P. falciparum* survival when monocytes had been stimulated with combinations of lentinan (L), parasite culture medium (c) and parasites (p) directly. Combinations of 10^5 monocytes/ml stimulated with 10μg/ml lentinan and 10^4 monocytes/ml stimulated with 20 μg/ml lentinan, were used in all subsequent experimentation.

As a general trend in the monocyte stimulation experiments (Figs 3.5 - 3.10), the unstimulated monocytes inhibited parasite survival to the least extent but an inhibition of ~ 50% did occur. This trend was expected since it has been reported that "macrophages and monocytes in culture on adherent surfaces may spontaneously elaborate IL-1" (Oppenheim, *et al.,* 1979). The greatest inhibition of parasite growth (20% survival) was noted after

stimulation of the monocytes with parasite culture medium, followed by stimulation of the monocytes by the parasites directly. A possible reason for this could be that the parasites released soluble cytotoxic substances into the culture medium , which were able to accumulate for either 12 or 24 hours depending on the experiment). The direct presence of the parasites was obviously not as stimulatory for the monocytes.

When 12 hour supernatants were used, a general decrease in parasite survival (greater inhibition of parasite growth) was observed. This could indicate that the cytokines produced by the monocytes are more stable and more effective early after their release into the supernatant, and that their activity decreases with time in culture. This explanation is supported by the findings of Fruehauf *et al.*(1982) who reported that the cytokines produced by monocytes , remain stable for ~ 21 hours in culture with a subsequent decline in effect up to 61 hours.

When the supernatants were heat inactivated, a general increase in percent parasite survival was observed. Since cytokines are glyco- or poly-peptide molecules, this increase can be explained by a denaturation of the protein structure of the molecules, with a subsequent decrease in effectiveness .

Surprisingly, when lentinan was added as an additional monocyte stimulant in all cases, the percent parasite survival was observed to increase instead of decrease. In other words, the lentinan did not stimulate the increased production of cytokines by the monocytes as anticipated. This finding appears to contradict *in vitro* experimentation undertaken by Fruehauf *et al.*(1982). They discovered that lentinan indeed causes the increased production of IL-1 by human monocytes *in vitro* as indicated by thymocyte proliferation in response to IL-1.

A possible explanation for the failureof lentinan to inhibit *P. falciparum* growth *in vitro* is as follows:

The failure of inhibition was probably not due to a lack of IL-1 production, since Freuhauf's experimentation described above indicated that IL-1 is indeed produced following stimulation with lentinan *in vitro*. The problem arose however , when the IL-1 which was produced, was unable to work on *P. falciparum* directly. The mechanism of action of IL-1 in the presence of an antigen, is to induce the production of IL-2 from T-cells. The IL-2 is a second mitogenic signal which causes proliferation of T-cells that have already encountered the antigen as the first signal, includind helper T-cells which have themselves produces IL-2. It is the IL-2 along with other bioactive substances that potentiaite the induction of different effector cells such as killer T-cells, NK cells and cytotoxic

macrophages as a result of sequential reactions (Chihara, 1992). It is these activated cells which would be able to inhibit *P. falciparum* growth *in vitro*.

It was therefore imperative to continue investigation into this compound using a more complete immune model - that is, a model which included not only monocytes, but T-cells as well..

3.6 CONCLUSIONS

* The immunostimulating polysaccharide lentinan has no direct effect on *P. falciparum in vitro*
* Supernatants from the monocytes stimulated *in vitro*, decrease parasitaemia depending on the stimulating agent, but lentinan had no stimulatory effect on the monocytes *in vitro*.
* It would be beneficial to determine the nature of the antimalarial compounds produced by the stimulated monocytes.

4. CHAPTER FOUR

RABBIT MONOCYTE EXPERIMENTS WITH LENTINAN : BRIDGE BETWEEN *IN VITRO* AND *IN VIVO* WORK

4.1 INTRODUCTION

Previous experimentation (CHAPTER 3) indicated that monocytes cultured *in vitro* produce substances which decrease *Plasmodium falciparum* survival to about 50% *in vitro*. This effect was enhanced by stimulating the cultured monocytes with various agents. Lentinan however, did not stimulate the monocytes *in vitro* and did not enhance the production of cytotoxic substances by the monocytes when combined with other stimulating agents.

Since lentinan is an immunomodulating agent, the next logical step was therefore to attempt to stimulate the monocytes *in vivo* prior to carrying out *in vitro* experimentation i.e. testing the supernatant effect of *in vivo* stimulated monocytes on *Plasmodium falciparum* survival, and compare these results with existing results.

Rabbits were used as the *in vivo* model for this section of experimentation with lentinan.

4.2 AIMS

* To determine the most effective method for maximum, uncontaminated monocyte retrieval from rabbits.

* To determine the effect on *P. falciparum* of supernantants from human monocytes stimulated with lentinan *in vitro* and cultured in medium containing fetal calf serum, human serum and serum free medium.

* To determine the effect on *P. falciparum* of supernantants from rabbit monocytes stimulated with lentinan *in vivo* and cultured in medium containing fetal calf serum, human serum and serum free medium .

4.3 METHODOLOGY

The following animal ethics clearance numbers were obtained from the Central Animal Services Unit at the University of the Witwatersrand for this section of experimentation: **92 138 3 ; 93 25 3**

All animal experimentation was carried out in two stages:

(i) Injection of saline or lentinan into the rabbit (*in vivo* part)

(ii) Isolation of monocytes from whole blood with subsequent *in vitro* experimentation using monocyte supernatants.

4.3.1 DETERMINATION OF THE EFFECT ON *P. FALCIPARUM* OF SUPERNATANTS FROM RABBIT MONOCYTES STIMULATED *IN VIVO* AND/OR *IN VITRO* WITH LENTINAN

3 Rabbits (Male or Female), strain SD, with body mass of ± 3 kgs were used. 2 Rabbits were used for experimental purposes, and 1 as a control.

The experimental animals were administered 1mg/kg (1.5 ml) lentinan via the ear vein. The control rabbit was administered 1.5 ml normal saline i.v. 24 Hours post injection, the rabbits were weighed and anaesthetised with 9mg/kg alphadolone and alfaxolone (Saffan) i.v. Blood was collected in heparinised tubes via a cardiac puncture. The animals were then euthanased with sodium pentobarbitone (100mg/kg i.v).

Monocytes were isolated according to the procedure outlined in section 2.3.1, and counted using a haemocytometer: $\sim 7 \times 10^6$ cells were retrieved from both the control and experimental animals.

The monocytes were then divided into four groups for experimental purposes:

(i) **C -** : Monocytes retrieved from control rabbit (not stimulated with lentinan *in vivo*)and not stimulated with lentinan *in vitro* either.

(ii) **C +** : Monocytes retrieved from control rabbit (not stimulated with lentinan *in vivo*), but thereafter stimulated with 10µg/ml lentinan *in vitro*. The monocytes were stimulated *in vitro* for 24 hours.

(iii) **V - :** Monocytes retrieved from experimental rabbit stimulated with 1mg/kg lentinan *in vivo*, but not stimulated with lentinan *in vitro*.

(iv) **V +** : Monocytes retrieved from experimental rabbit stimulated with 1 mg/kg lentinan *in vivo*, and thereafter stimulated with 10µg/ml lentinan *in vitro*. The monocytes were stimulated *in vitro* for 24 hours.

The monocytes were left in culture for 24 hours, centrifuged at 1500 rpm and the supernatants stored at -20^0C until use in the hypoxanthine assays which were carried out according to 2.2. and repeated for verification of results.

Each combination (C-, C+, V-, V+) was tested in repeats of 30.

4.3.2 DETERMINATION OF THE MOST EFFECTIVE RABBIT MONOCYTE RETRIEVAL METHOD

Monocytes retrieved from one control rabbit were used in experimentation to determine the best method for maximum monocyte retrieval.

4.3.2.1 Experimental procedure *in vivo*

The rabbit was administered 1.5 ml of normal saline via the ear vein, once daily for 3 days. On Day 4, the rabbit was anaesthetised and ± 60 mls blood was collected via a cardiac puncture. The rabbit was then euthanased.

4.3.2.2 Experimental procedure *in vitro*

The monocytes were separated according to the procedure in section 2.3.1 , but the following three modifications were made:

Method 1:

1. The whole blood was not diluted prior to separation. The blood was spun down in a small centrifuge at 1900rpm and 1500rpm.

Method 2:

2. The whole blood was not diluted prior to separation. The blood was spun down in a large centrifuge at 1400rpm and 1150rpm.

Method 3:

3. The whole blood was diluted 1:2 with PBS prior to separation. The blood was spun down in a large centrifuge at 1250rpm and 1000rpm.

The monocytes were then counted , placed into 3 glass petri dishes and cultured for 24 hours. Half of the supernatants were stored in the fridge (at -4° C) and the other half in the freezer (at -20° C) until use in the hypoxanthine assays which were carried out according to procedures described in section 2.2.1.

4.3.3 THE *IN VITRO* EFFECT ON *P. FALCIPARUM* OF SUPERNATANTS FROM HUMAN MONOCYTES CULTURED IN FETAL CALF, SERUM FREE AND HUMAN SERUM AND STIMULATED WITH LENTINAN.

Before *in vivo* experimentation could be continued, the next step was to determine whether or not the fetal calf serum in the monocyte culture medium affected parasite survival *in vitro*. Simultaneously, the effect of lentinan at varying concentrations on the monocytes was investigated.

Three stocks of lentinan were prepared for the experiment: 10μg, 1μg and 0.1μg, according to section 2.2.2.3. Human whole blood was used for these experiments. The monocytes were isolated according to procedures described in section 2.3.1 and counted using a haemocytometer. The total cell count was ~ 10^7 cells/ml .

9 x 10^5 monocytes/ml were then plated out in each plate as follows :

[A] : **MONOCYTES CULTURED IN HUMAN SERUM (HS)**

A1 Monocyte control

A2 : Monocytes stimulated with 1μg/ml lentinan (final concentration)

A3 : Monocytes stimulated with 0.1μg/ml lentinan (final concentration)

A4 : Monocytes stimulated with 0.01μg/ml lentinan (final concentration)

[B] : **MONOCYTES CULTURED IN FETAL CALF SERUM (FCS)**

PLATES B1 - B4 : Plates set up as in [A] above, with 9×10^5 cells/ml in each plate

[C] : **MONOCYTES CULTURED IN SERUM FREE MEDIUM (SF)**

PLATES C1 - C4 : Plates set up as in [A] above, with 9×10^5 cells/ml in each plate

A hypoxanthine assay was then performed out according to section 2.2.

4.3.4 THE *IN VITRO* EFFECT ON *P. FALCIPARUM* OF SUPERNANTANTS FROM RABBIT MONOCYTES CULTURED IN HUMAN SERUM AND FETAL CALF SERUM

This experiment was carried out in order to compare the effect on *P. falciparum* of using fetal calf serum and human serum in the monocyte culture medium of the rabbit monocytes as had been done in the *in vitro* human monocyte work (section 4.3.3). Also, since the new stock of lentinan had to be dissolved in a mannitol/dextran/distilled water solution, the rabbit was injected with this solution instead of normal saline.

The rabbit was inoculated via the ear vein 1 x daily for 3 days with 1.5ml of the dextran/mannitol solution. On Day 4, the rabbit was anaesthetised, exsanguinated (blood was collected in 6 x 10ml tubes) and then euthanased.

Monocytes were retrieved according to Method #3 (section 4.3.2.2 above), counted using a haemocytometer (~ $1x\ 10^6$ cells/ml were counted), and then plated out according to the following :

A1: Monocytes cultured in medium containing human serum

B1: Monocytes cultured in medium containing fetal calf serum

The cells were left in culture for 24 hours and the supernatants were then tested on the parasites via a hypoxanthine assay which was carried out according to section 2.2.

4.3.5 THE *IN VITRO* EFFECT ON *P. FALCIPARUM* OF SUPERNATANTS FROM RABBIT MONOCYTES STIMULATED *IN VIVO* WITH LENTINAN AND CULTURED *IN VITRO* IN HUMAN SERUM, FETAL CALF SERUM AND SERUM FREE MEDIUM

In this experiment involving rabbit monocytes, the effect on *P. falciparum* of supernatants from monocytes cultured in medium containing human serum, fetal calf serum and serum free medium was assessed. The effect of stimulating the monocytes *in vivo* with lentinan prior to retrieval was also assessed.

The control (untreated rabbit) was administered 2ml of a mannitol/dextran solution in distilled water i.v. once daily for 3 days. The experimental rabbit was administered 1mg/kg lentinan i.v. in a mannitol/dextran solution also once daily for 3 days in order to stimulate the monocytes *in vivo*. The rabbits were then anaesthetised, exsanguinated (± 6 x 10mls of blood was obtained from each rabbit) and then euthanased.

The monocytes were separated out from the whole blood, counted using a haemocytometer (~ 3 x 10^6 cells/ml were counted for both the experimental and control animals) and then plated out at various concentrations (C1, C2 and C3) as follows:

(a) **Control Plates:**

C1:	(i)	HS	**C1:**	(i)	HS	**C3:**	(i)	HS
	(ii)	FCS		(ii)	FCS		(ii)	FCS
	(iii)	SF		(iii)	SF		(iii)	SF

Where:

C1	=	final cell concentration of ~ 1×10^6 cells/ml
C2	=	final cell concentration of ~ 0.5×10^6 cells/ml
C3	=	final cell concentration of ~ 0.1×10^6 cells/ml
HS	=	monocytes cultured in medium containing human serum
FCS	=	monocytes cultured in medium containing fetal calf serum
SF	=	monocytes cultured in serum free medium

(b) **Experimental plates:**

The experimental plates were set up in exactly the same manner as the control plates above. The cells were left in culture for 24 hours and the supernatants were then tested on the parasites via a hypoxanthine assay which was carried out according to section 2.2.

4.4 RESULTS

4.4.1 THE EFFECT ON *P. FALCIPARUM* OF SUPERNATANTS FROM RABBIT MONOCYTES STIMULATED *IN VIVO* AND/OR *IN VITRO* WITH LENTINAN

Figure 4.1 shows that there was no difference in percent parasite survival when *in vivo* lentinan stimulated or unstimulated rabbit monocytes were cultured *in vitro* with or without lentinan.

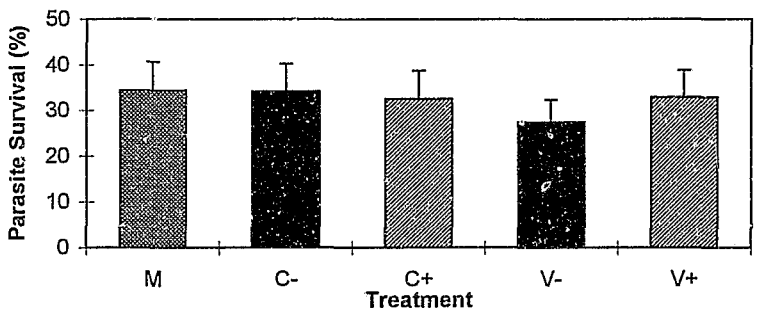

Figure 4.1: The effect on *P. falciparum* of various 24 hour monocyte supernatants

Where:

M = Monocyte culture medium control

C- = Supernatants from monocytes stimulated neither *in vitro* nor *in vivo* with lentinan

C+= Supernantants from monocytes stimulated *in vitro*, but not *in vivo* with lentinan

V- = Supernantants from monocytes stimulated *in vivo*, but not *in vitro* with lentinan

V+= Supernatants from monocytes srtimulated both *in vivo* and *in vitro* with lentinan

4.4.2 DETERMINATION OF THE MOST EFFECTIVE RABBIT MONOCYTE RETRIEVAL METHOD

The monocyte counts for the various modified methods used to determine the maximum yield of cells are listed in Table 4.1. Although the number of cells retrieved was very similar for each technique used (~ 10^6 cells/ml in each case), Method #3 resulted in the lowest degree of monocyte contamination with erythrocytes and the lowest extent of erythrocyte lysis. There was also no difference in effect on parasite survival when monocyte supernatants were stored at -4°C or at -20°C prior to use in the hypoxanthine assay (Table 4.2). The percent parasite survival was ~ 40% in all cases.

Table 4.1: Rabbit monocyte counts for the various separation techniques

MONOCYTE SEPARATION TECHNIQUE	CELL COUNT
Method # 1	1.7 x 10^6 cells/ml
Method # 2	3.2 x 10^6 cells/ml
Method # 3	1.4 x 10^6 cells/ml

Table 4.2: The effect of supernatants from rabbit monocytes stored in the fridge (at -4° C) and the freezer (at -20° C) on *P. falciparum* survival. Monocytes were retrieved via 3 different methods.

SEPARATION TECHNIQUE	% PARASITE SURVIVAL (Supernatant stored at -4°C)	% PARASITE SURVIVAL (Supernatant stored at -20°C)
Control	36.8 ± 4.4	39.4 ± 9.4
Method # 1	39.2 ± 5.9	35.8 ± 6.9
Method # 2	35.1 ± 8.6	32.7 ± 5.1
Method # 3	37.1 ± 8.9	33.8 ± 3.9

4.4.3 THE *IN VITRO* EFFECT ON *P. FALCIPARUM* OF SUPERNATANTS FROM HUMAN MONOCYTES CULTURED IN FETAL CALF SERUM, HUMAN SERUM AND SERUM FREE MEDIA STIMULATED WITH LENTINAN

Figure 4.2 shows that there was no difference in percent parasite survival when supernatants are used from human monocytes cultured in human serum or serum free medium (survival is ~ 50 in the lentinan non-stimulated monocyte controls). Parasite survival decreased however, when supernatants are used from monocytes cultured in fetal calf serum (survival is reduced to ~25% controls). It is also evident that lentinan at various concentrations has no effect on parasite survival in any of the media.

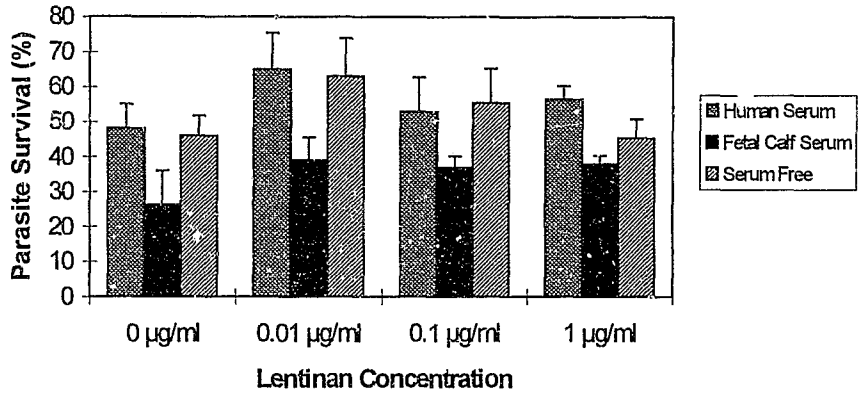

Figure 4.2: Hypoxanthine assay results for human monocytes cultured in fetal calf serum, human serum and serum free media, stimulated with varying concentrations of lentinan

4.4.4 THE *IN VITRO* EFFECT ON *P. FALCIPARUM* OF SUPERNATANTS FROM RABBIT MONOCYTES CULTURED IN HUMAN SERUM AND FETAL CALF SERUM.

When the *in vitro* effect of supernatants from rabbit monocytes cultured in human serum versus fetal calf serum was tested (Table 4.3), the percent parasite survival was once again much lower in the fetal calf serum group (~57%) compared to the human serum group (~90%).

Table 4.3: The effect on *P. falciparum* of supernatants from rabbit monocytes cultured *in vitro* in with medium containing fetal calf serum and human serum

GROUP	SERUM IN CULTURE MEDIUM	% PARASITE SURVIVAL (MEAN ± SD)
A1	Fetal calf serum	57.5 ± 9.6
B1	Human serum	90.9 ± 8.4

4.4.5 THE *IN VITRO* EFFECT ON *P. FALCIPARUM* OF SUPERNATANTS FROM RABBIT MONOCYTES STIMULATED *IN VIVO* WITH LENTINAN AND CULTURED *IN VITRO* IN HUMAN SERUM, FETAL CALF SERUM AND SERUM FREE MEDIA

When the effect on *P. falciparum* of supernatants from lentinan unstimulated (control) or lentinan stimulated (*in vivo* stimulated) rabbit monocytes was investigated, the percent parasite survival was similar when the monocytes were cultured in human serum or serum free medium (~80%), but much lower when the monocytes were cultured in fetal calf serum (~50%) . Stimulating the monocytes *in vivo* with lentinan at various concentrations prior to retrieval, had no effect on decreasing parasitaemia in either of the three case (Figure 4.3).

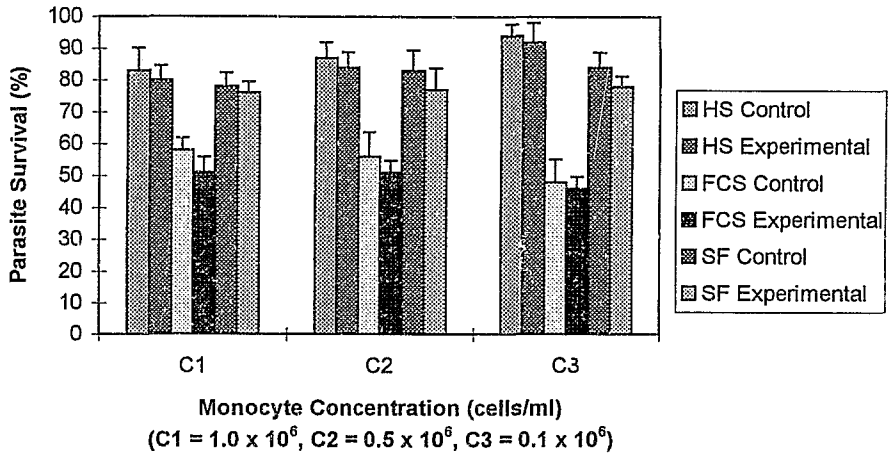

Figure 4.3: The effect on *P. falciparum* of various supernatants from rabbit monocytes stimulated *in vivo* with lentinan. Experimental rabbits administered 1 mg/kg lentinan.

Where:

HS = Monocytes cultured in human serum

FCS = Monocytes cultured in fetal calf serum

SF = Monocytes cultured in serum free medium

C1 = 1.0×10^6 monocytes/ml

C2 = 0.5×10^6 monocytes/ml

C3 = 0.1×10^6 monocytes/ml

4.5 DISCUSSION

With reference to Table 4.1, no apparent differences in parasite survival were found between any of the rabbit monocyte groups compared to the control (monocyte medium) i.e. parasite survival was ~ 30% in all cases.

However, since there was a certain degree of contamination of the monocytes with erythrocytes and a certain degree of blood lysis during the monocyte separation procedure, it was necessary to repeat the experiment taking the following changes into account:

(i) Trying various monocyte separation techniques in order to determine the method most effective method for maximum uncontaminated monocyte retrieval .

(ii) Making changes in the monocyte culture medium itself: i.e testing the differences in effect on the parasite when supernatants are used from monocyte medium prepared with Fetal calf serum, Human Serum or in Serum free medium.

(iii) Stimulating the rabbits with lentinan for a longer period of time prior to monocyte rerieval.

Table 4.1 shows the results for the various monocyte separation techniques which were tried in order to attain maximum concentrations of uncontaminated monocytes. Although the number of cells retrieved was very similar for each of the three methods (10^6 cells/ml in each case), Method #3 (refer to section 4.3.2.2), resulted in the lowest degree of monocyte contamination with erythrocytes and the smallest degree of erythrocyte lysis. This method was therefore used in all ensuing experimentation.

With reference to Table 4.2, there was no difference in effect on parasite survival when monocyte supernatants were stored at -4°C or at -20°C prior to use in the hypoxanthine assay (% parasite survival was ~ 40% in all cases). The 40% parasite survival rate in the control (monocyte culture

medium alone),was however of great concern. Parasite survival should have been ± 100%. A possible explanation could be that the parasites which are cultured in media containing human serum, reacted adversely to the fetal calf serum used in the monocyte culture medium, which was later present in the monocyte supernatants used in the hypoxanthine assays. The next logical step was therefore to test the effect on *P. falciparum* of monocytes cultured in media containing various sera.

With reference to Figure 4.2, percent parasite survival was similar when the human monocytes used at this stage of experimentation, were cultured in either serum free medium , or medium containing human serum. The lentinan at three varying concentrations, had no effect in decreasing percent parasite survival compared to the controls. In fact, parasite survival increased when lentinan at all three concentrations was tested. A decrease in percent parasite survival was observed however , when the monocytes were cultured in fetal calf serum. Survival was ~25% compared to ~ 50 % in the human serum and serum free groups. Since the lentinan at varying concentrations, also had no effect in decreasing parasite survival in the fetal calf serum group(a slight increase was once again observed with increasing concentrations), the decrease was solely due to the fetal calf serum being present in the monocyte supernatants.

The effect on *P. falciparum* survival of the fetal calf serum compared to human serum was then tested on monocytes retrieved from the second control rabbit. Table 4.3 again shows the effect of supernatants from rabbit monocytes cultured in fetal calf serum compared to human serum on the parasites - survival of the parasites was 57.5% in the fetal calf serum group compared to 90.9% in the human serum group.

The final experiment involving rabbit monocytes, compared the effect on *P. falciparum* of supernatants from unstimulated (control) and *in vivo* stimulated (experimental) monocytes, cultured *in vitro* with medium containing human serum, fetal calf serum and serum free medium. The above results were once again confirmed in Figure 4.3. The percent parasite survival was similar when monocytes were cultured in human serum or serum free medium (~ 80%) in both the control and experimental groups, at varying monocyte concentrations. Stimulating the monocytes *in vivo* with lentinan, also had no effect in decreasing parsitaemia compared to the controls. Culturing the monocytes in fetal calf serum once again decreased percent parasite survival to ~ 50% in both the control and experimental groups. Stimulating the monocytes *in vivo* with lentinan prior to retrieval, also had no effect in decreasing parasite survival compared to the controls.

Since the lentinan did not have an effect in stimulating the rabbit monocytes *in vivo* (no *in vitro* effect on *P. falciparum* was observed when supernatants from these monocytes were used), the next and final step was therefore to test the effect of the drug completely in an *in vivo* model. The effect of the lentinan on *Plasmodium berghei* infection in mice was therefore carried out.

4.6 CONCLUSIONS

* There is no difference in percent parasite survival when supernatants are used from human or rabbit monocytes cultured either in human serum or in serum free medium. The parasitaemia decreases however when the monocytes are cultured in fetal calf serum.

* Lentinan does not have a stimulatory effect on rabbit monocytes either *in vitro* or *in vivo*.

5. CHAPTER 5 - *IN VIVO* MICE EXPERIMENTS WITH LENTINAN AND *PLASMODIUM BERGHEI*

5.1 INTRODUCTION

As a host defence potentiator, lentinan has been shown to augment host defence mechanisms through the host immune system, and is active against various kinds of infectious diseases and cancer (Chihara 1992). It has been tested in humans for the treatment of immunodeficiencies, especially AIDS and in cancer patients. The average dose in humans is from 0.1 - 10mg/kg reaching levels of 1 - 100 μg/ml. The following adverse effects have been described in humans (incidence 6.8%) : eruption and redness, nausea and vomiting, headache, sweating, dizziness, etc (Taguchi , 1985). Lentinan has no CNS side effects on tested rats (Masek, 1990).

Antiparasitic activity has been reported for several polysaccharides. They act by enhancing the host's resistance against the parasites, dependant on cell-mediated immunity. Yeast glucan (a polysaccharide similar to lentinan) was reported to prolong survival in *Plasmodium berghei* infected mice (Song , 1979). Since previous experimentation (refer to CHAPTERS 3 & 4), showed that lentinan has no antimalarial effect *in vitro,* it was imperative to carry out complete *in vivo* studies in mice in order to fully evaluate the efficacy of the immunopotentiating drug lentinan in malaria infection. These *in vivo* investigations were carried out in Balb/c mice model using *Plasmodium berghei* as the infective agent.

5.2 AIMS

* To detect the tolerance of lentinan at 10mg/kg/day
* To develop experimental inoculum of *Plasmodium berghei* in Balb/c mice
* To investigate the effect of lentinan on *P.berghei* infection in mice
* To investigate the effect of pre-treatment with lentinan on malaria infection in mice
* To investigate the effect of *P.berghei* inoculum size on malaria infection in mice

5.3 METHODOLOGY

The following animal ethics clearance number was obtained from the Central Animal Services Unit (CASU) at the University of the Witwatersrand for this section of experimentation: **92/104/4**

5.3.1 DETECTING THE TOLERANCE OF LENTINAN AT A DOSE OF 10 MG/KG ADMINISTERED DAILY FOR 8 DAYS.

Number of mice required : 10 Female Balb/c mice

5 Mice were administered 0.5ml lentinan intraperitonealy (i.p) , daily for 8 days, at a dose of 10mg/kg. 5 Control mice were administered normal saline 0.5ml i.p daily. All of the animals were weighed on a daily basis. On Day 8, the animals were anaesthestised (as described in section 2.4.1.3), blood samples obtained for haematocrit calculation (as described in section 2.4.1.5), and then euthanased (as described in section 2.4.1.3).

5.3.2 DEVELOPMENT OF EXPERIMENTAL INOCULUM OF *PLASMODIUM BERGHEI*

The procedure for inoculum development was carried out as per section 2.4.1.3.

5.3.3 THE EFFECT OF LENTINAN AT 10 MG/KG I.V. ON MALARIA INFECTION IN MICE

Number of mice required: 10 Female Balb/c mice

The 10 mice were divided into 2 groups - 1 Experimental and 1 Control . On Day 0, they were each infected i.p. with 0.1ml *P.berghei* inoculum (administration of 10^6 parasitised erythrocytes). From Day 1, treatment of the 5 experimental animals with lentinan commenced. The experimental animals were administered 10mg/kg i.v. into the tail vein , once daily for 7 days. The control animals were administered a saline placebo i.v. into the tail vein, once daily for 7 days. The minor change in drug administration procedure from i.p to i.v directly was made (and approved by the CASU) due to our concern over the absorption of polysaccharides from the peritoneal cavity into the system. The animal weights and parasitaemias were monitored daily (as per sections 2.4.2.1 and 2.4.2.2). The last dose of lentinan was administered on Day 6. The animals were then anaesthetised (as per section 2.4.1.3) and bled from the tail vein in order to obtain blood for haematocrit calculation (as per section 2.4.1.5). The animals were then euthanased (as per section 2.4.1.3) and their spleens surgically removed and weighed in order to calculate spleen indices (as per section 2.4.1.6).

5.3.4 PRE-TREATMENT OF EXPERIMENTAL ANIMALS WITH LENTINAN AT 10MG/KG I.V. PRIOR TO INOCULATION WITH *P.BERGHEI*

Number of mice required: 10 Female Balb/c mice

The 10 mice were divided into 2 groups - 1 Experimental and 1 Control . From Day 0 (the first day of drug/saline administration), pre-treatment of the 5 experimental animals with lentinan commenced. The animals were administered 10mg/kg i.v. into the tail vein , once daily for 6 days (Day 0 - Day 5). The control animals were administered a saline placebo i.v. into the tail vein, once daily for 6 days (Day 0 - Day 5). From Day 6, treatment with lentinan and the placebo was stopped. On Day 6, all 10 animals were infected i.p. with 0.1ml *P.berghei* inoculum (administration of 10^6 parasitised erythrocytes) The animal weights and parasitaemias were monitored daily (as per sections 2.4.2.1 and 2.4.2.2).. The animals were then anaesthetised (as per section 2.4.1.3) and bled from the tail vein in order to obtain blood for haematocrit calculation (as per section 2.4.1.5). The animals were then euthanased (as per section 2.4.1.3) and their spleens surgically removed and weighed in order to calculate spleen indices (as per section 2.4.1.6).

5.3.5 EXPERIMENTATION WITH LENTINAN AT 10MG/KG I.V. - INOCULATION OF MICE WITH 10^3 INSTEAD OF 10^6 PARASITES IN ORDER TO PROLONG SURVIVAL TIME.

Number of mice required: 20 Female Balb/c mice

The 20 mice were divided into 4 groups - 2 Experimental and 2 Control. On Day 0, they were each infected i.p. with 0.1ml *P.berghei* inoculum (administration of 10^3 parasitised erythrocytes). From

Day 1, treatment of the 10 experimental animals with lentinan commenced. The experimental animals were administered 10mg/kg lentinan into the tail vein, once daily. The control animals were administered a placebo i.v. into the tail vein, once daily. The animals' weights and parasitaemias were monitored daily. The last dose of lentinan was administered up until Day 14 (depending on the animals' survival rate). The "end day" varied for the mice from Day 11 to Day 14. The animals were then anaesthetised (see section 2.4.1.3) and bled from the tail vein in order to obtain blood for haematocrit calculation. The animals were then euthanased (see section 2.4.1.3) and their spleens surgically removed and weighed in order to calculate spleen indices (see section 2.4.1.6).

5.3.6 STATISTICAL ANALYSIS

The results of the parasitaemia were expressed as the Mean ± SD. The statistical significance of the difference between the control and the CRDS treated groups was evaluated using the Student's *t* test. The difference was considered significant when $P < 0.05$.

5.4 RESULTS

5.4.1 LENTINAN TOLERANCE TRIAL

Lentinan at 10mg/kg, administered daily for 8 days was well tolerated by the experimental animals, since it had no effect on either animal weight (Table 5.1) or haematocrit (Table 5.2) compared to the control animals.

Table 5.1: Mean ± SD of weights for the experimental and control animals on Days 1 and 8.

	Mean weight (grams) ± SD	
	Day 1	**Day 8**
Experimental (lentinan treated)	17.60 ± 0.55	18.40 ± 0.55
Control (Saline)	18.00 ± 0.71	18.25 ± 0.50

Table 5.2 : Mean ± SD of Haematocrit values for the Experimental and Control animals on Day 8

	Mean Haematocrit ± SD (%)
Experimental	49.80 ± 2.86
Control	50.00 ± 1.63

5.4.2 THE EFFECT OF LENTINAN AT 10MG/KG I.V. ON MALARIA INFECTION IN MICE

Lentinan administered at a dose of 10mg/kg/day for 7 days, had no effect on the weight of the experimental animals compared to the controls (Table 5.3). The mean "end day" (as defined in

section 2.4.1.5) parasitaemia was slightly lower in the experimental animals (~50%) compared to the controls (~ 60%), but the lentinan failed to be effective in clearing parasitaemia in the mice (Figure 5.1). With reference to Tables 5.4 & 5.5, both the mean spleen indices and haematocrits were lower in the experimental animals compared to the controls.

Table 5.3: Mean ± SD of daily weights for experimental (lentinan treated) and control mice

	Daily weight (grams)	
Day	**Experimental**	**Control**
0	20.9 ± 0.71	19.8 ± 1.25
1	21.2 ± 0.80	20.9 ± 1.50
2	19.8 ± 0.91	19.7 ± 1.71
3	20.2 ± 0.84	19.7 ± 1.54
4	20.5 ± 0.65	19.2 ± 1.81
5	19.3 ± 0.89	18.2 ± 1.49
6	18.1 ± 0.73	16.9 ± 1.48

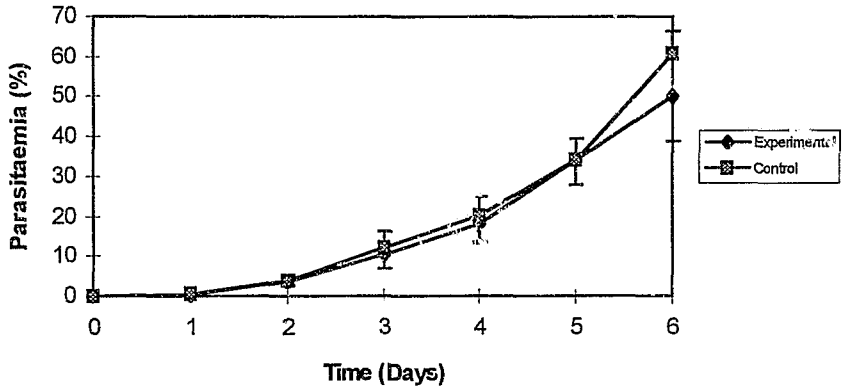

Figure 5.1: Mean ± SD of daily parasitaemias for experimental (lentinan treated) and control mice

Table 5.4: Mean ± SD of Spleen Indices for experimental and control mice at "end day"

Spleen Index	
Experimental	**Control**
1.7 ± 0.21	2.23 ± 1.38

Table 5.5: Mean ± SD of Haematocrits for experimental and control mice at "end day"

Haematocrit	
Experimental	**Control**
35.3 ± 2.48	44.0 ± 2.12

5.4.3 THE EFFECT OF PRE-TREATMENT WITH 10MG/KG LENTINAN FOR 6 DAYS ON THE WEIGHT OF EXPERIMENTAL ANIMALS

Pre-treatment of the experimental animals with lentinan had a minimal effect on their weight compared to the controls (Table 5.6). Figure 5.2 shows that the mean "end day" parasitaemia (defined in section 2.4.1.5) of the experimental animals was only slightly lower (53.6 %) than that of the controls (58.3%), as was their mean spleen index (Table 5.7)

Table 5.6: Mean ± SD of Daily weights for experimental (lentinan pre-treated) and control mice

	Daily weight (grams)	
Day	**Experimental**	**Control**
0	19.6 ± 0.88	19.6 ± 0.64
1	20.4 ± 0.93	20.7 ± 0.82
2	20.1 ± 0.95	20.5 ± 0.83
3	20.4 ± 0.80	20.4 ± 0.72
4	20.9 ± 0.98	20.7 ± 0.35
5	20.7 ± 0.95	20.4 ± 0.65
6	20.9 ± 0.92	21.0 ± 0.68
7	20.7 ± 1.02	20.5 ± 0.60
8	19.3 ± 1.17	19.4 ± 0.69
9	19.3 ± 0.97	19.6 ± 0.94
10	19.9 ± 0.83	19.9 ± 0.57
11	18.4 ± 1.06	18.8 ± 1.13
12	17.2 ± 0.89	17.5 ± 1.01

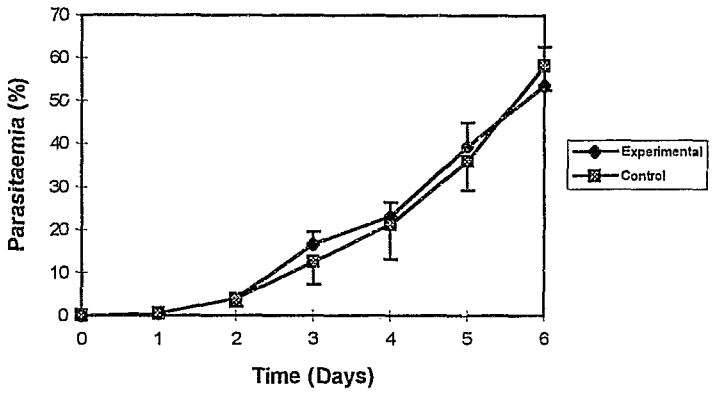

Figure 5.2: Mean ± sd of daily parasitaemias for experimental (lentinan pre-treated) and control mice

Table 5.7: Mean ± SD of Spleen Indices for experimental (pre-treated) and control mice at "end day"

Spleen Index	
Experimental	**Control**
1.6 ± 0.21	1.75 ± 0.12

5.4.4 EXPERIMENTATION WITH LENTINAN AT 10MG/KG I.V. - INOCULATION OF MICE WITH 10^3 INSTEAD OF 10^6 PARASITES IN ORDER TO PROLONG SURVIVAL TIME.

Lentinan did not affect the extent of weight loss in either the experimental or the control animals, even when fewer parasites were administered to the animals (Table 5.8). Figure 5.3 shows that parasitaemia development was similar in both the lentinan treated and control animals, with mean"end day" parasitaemias being almost exactly the same in both groups (59.5% in the

experimental compared to 58.2% in the controls). The mean spleen index was slightly lower in the experimental group compared to the control (Table 5.9), whereas the mean haematocrit was slightly higher in the experimental compared to the control group (Table 5.10).

Table 5.8: Mean ± SD of Daily weights for experimental (lentinan treated) and control mice

	Daily weight (grams)	
Day	**Experimental**	**Control**
0	17.6 ± 0.80	17.8 ± 0.94
1	17.7 ± 0.92	17.9 ± 1.03
2	16.9 ± 0.71	17.5 ± 1.18
3	17.2 ± 0.74	17.8 ± 1.27
4	17.3 ± 1.46	17.7 ± 1.34
5	17.7 ± 0.79	17.9 ± 1.08
6	17.7 ± 0.91	17.9 ± 1.05
7	17.4 ± 0.84	17.4 ± 1.15
8	16.9 ± 0.80	16.5 ± 1.05
9	16.3 ± 0.82	16.4 ± 1.60
10	15.8 ± 1.01	15.6 ± 1.52
11	15.5 ± 1.19	15.4 ± 1.45
12	15.0 ± 1.06	15.4 ± 1.17
13	14.2 ± 1.08	15.5 ± 0.31
14	14.4 ± 0.54	14.4 ± 1.00

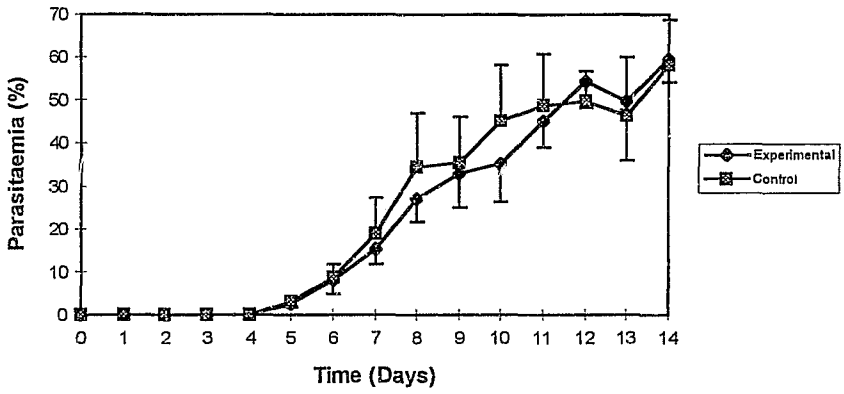

Figure 5.3: Mean ± SD of Daily parasitaemias for experimental (lentinan treated) and control mice

Table 5.9: Mean ± SD of Spleen Indices for experimental and control mice at "end day"

Spleen Index	
Experimental	**Control**
4.69 ± 0.74	4.92 ± 1.38

Table 5.10: Mean ± SD of Haematocrits for experimental and control mice at "end day"

Haematocrit	
Experimental	**Control**
25.0 ± 2.82	23.5 ± 3.12

5.5 DISCUSSION

The initial *in vivo* investigation step, was to carry out a tolerance study for the lentinan on the mice. Lentinan at a dose of 10mg/kg administered for 8 days was well tolerated in the experimental animals since it had no effect on either the weight (Table 5.1) or the haematocrit (Tables 5.2) of these animals in comparison to the control animals which were administered only normal saline.

Since the lentinan was not found to be toxic to the mice at 10 mg/kg/day, this was the dose used in all future experimentation with the mice and *Plasmodium berghei* infections.

In the first experiment where lentinan was administered to *P.berghei* infected mice, the lentinan had no effect on the mean weight of experimental animals (Table 5.3). The weight loss in the experimental animals (2.8 grams) compared to the controls (2.9 grams) was almost identical.

As far as parasitaemia development is concerned, although the lentinan failed to clear the parasitaemia in the mice, parasite multiplication was slightly retarded - the mean "end day" parasitaemia in the treated animals was ± 50% compared to ± 60% in the controls (Figure 5.1). This may have been due to slight stimulation of the host's immune system by the lentinan. The observed differences in parasitaemia development however, may not only have been due to the administration of the lentinan, but the differences may also have been influenced by the *P.berghei* inoculum itself and the accuracy of its intraperitoneal administration .

With reference to Tables 5.4 and 5.5, both the mean spleen index and the mean haematocrit were lower in the experimental animals compared to the controls. However, these haematocrit results may have differed from previous experimentation, because difficulties were experienced with the equipment for haematocrit determination .

The next step of the investigation was to determine the effect of pre-treating the experimental animals for 6 days with lentinan , prior to inoculation with *P.berghei*.

Pre-treatment with lentinan , did not affect their weight loss - the experimental animals lost 2.3 grams compared to a weight loss of 2.1 grams in the controls (Table 5.6). As far as parasitaemia is concerned (Figure 5.2), the lentinan did not appear to have any effect on parasitaemia compared to the controls. Although lower than in the control mice, the "end day" parasitaemia of the lentinan pre-treated animals was still high at 53.6%. The lentinan pre-treated mice however, did appear to show less severe clinical signs of disease (higher level of activity, better condition of fur and lighter coloured urine) than the control mice .

The mean spleen index in the experimental animals was lower than that of the controls (Table 5.7) - this followed the general trend of previous experimentation. Unfortunately, it was not possible to compare the mean haematocrits of the two groups, due to problems with equipment when collecting blood from the experimental animals .

In the final set of experimentation involving lentinan, fewer parasites (10^3 instead of 10^6) were administered to the animals . The mice were expected to survive 10-12 days instead of the usual 6 days. Since the rise in parasitaemia is a logarithmic function, parasitemia development at a slower rate was expected to suit these experiments involving the immune system better.

Extent of weight loss (Table 5.8) was once again very similar in both the lentinan treated and control groups - 3.2 grams for the treated animals compared to 3.4 grams for the controls. In this experiment, parasitaemia development was also very similar in both the experimental and the control groups. Parasites appeared at the same time (Day 4) in both groups and also developed at the same rate in both groups. If we examine the mean "end day" parasitaemias (Figure 5.3), there is once again very little difference between the two groups - 59.5% for the experimental group vs 58.2 % for

the control group. The lentinan did not appear to have any stimulatory effect on the host's immune system to aid in the clearing of parasites.

Table 5.9 indicates that the mean spleen index for the experimental animals was slightly lower in the experimental animals compared to the controls, whereas the mean haematocrit of the experimental animals was slightly higher than that of the controls (Table 5.10).

5.6 CONCLUSION

* Since lentinan is ineffective in clearing parsitaemia, or preventing parasite multiplication in *Plasmodium berghei* infection in mice, this drug does not appear to have a an immunomodulating effect *in vivo.*

6. CHAPTER SIX - *IN VITRO* EXPERIMENTATION WITH CURDLAN SULPHATE AND *PLASMODIUM FALCIPARUM*

6.1 INTRODUCTION

Lentinan and curdlan are both ß-1,3-D-glucans - mucopolysaccharides of similar chemical structure which stimulate the immune system non-specifically and which are currently being used in cancer and HIV patients (Chihara *et al*, 1970).

Since lentinan was ineffective in clearing parasitemias in previous *in vitro* experimentation (CHAPTERS 3 & 4), and in *in vivo* experiments (CHAPTER 5), the next step was to determine the effect of lentinan in its sulphated form against *P. falciparum in vitro* and *in vivo* . This hypothesis stemmed from the knowledge that certain fractions of another sulphated mucopolysaccharide, heparin, have been observed to inhibit the invasion of *P. falciparum* merozoites *in vitro* (Carlson *et al*, 1992) . The pronounced anticoagulant effect and increased risk of bleeding in patients administered with heparin (Levine *et al*, 1989) however, excludes its use as an anticytoadherent agent. In their sulphated forms, lentinan and curdlan have a much less severe anticoagulant effect than heparin, but they are still able to prevent cytoadherence e.g. curdlan sulphate has exhibited potent blocking effects on the binding of HIV-1 virions to the surfaces of target CD4+ H9 cells) *in vitro* without serious toxicity (Aoki *et al*, 1991; Aoki *et al*, 1992).

We proposed that lentinan sulphate or curdlan sulphate may similarly inhibit the binding to and subsequent invasion of erythrocytes by *Plasmodium* merozoites. In this manner, parasite multiplication within the erythrocyte would then be prevented and the parasitaemia *in vitro* and *in vivo* in the host would eventually decrease.

Since trials had previously been conducted with lentinan in its non sulphated form, it would have been obvious to compare it to lentinan in its sulphated form. However, having recently been

supplied with curdlan sulphate from the Ajinomoto Company in Japan , the investigation into sulphated polysaccharides was continued using this related compound instead.

6.2 AIMS

* To determine the *in vitro* effect of curdlan sulphate on *Plasmodium falciparum* over a single and double cycle of parasite growth.
* To determine the *in vitro* effect of curdlan sulphate in combination with the classical antimalarials: quinine, chloroquine and mefloquine on *P. falciparum*.

6.3 METHODOLOGY

6.3.1 ASSESSING THE EFFECT OF CURDLAN SULPHATE ON *PLASMODIUM FALCIPARUM* OVER A SINGLE AND DOUBLE CYCLE OF GROWTH

6.3.1.1 Preparation of the stock solution of curdlan sulphate (CRDS) for the Hypoxanthine Assay

A 50000 µg/ml CRDS stock solution and 32 serial ¾ dilutions were then prepared. Since all drugs are diluted 1:10 in the IC_{50} plate of a hypoxanthine assay, the first actual concentration of CRDS to be tested in the plate was 5000µg/ml, with a final concentration of 0.7µg/ml.

The Hypoxanthine Assay was then carried out as per section 2.2 and the IC_{50} values of the curdlan sulphate were determined over single and double cycles of growth.

6.3.2 ASSESSING THE EFFECT OF THE CLASSICAL ANTI-MALARIALS QUININE, CHLOROQUINE AND MEFLOQUINE IN COMBINATION WITH CRDS ON THE *P. FALCIPARUM* RESISTANT (FCR-3) AND SENSITIVE (3D7) STRAINS

6.3.2.1 CRDS in combination with quinine on the *P. falciparum* 3D7 strain

(i) Preparation of the quinine and curdlan sulphate stock solutions:

A 400nM (20x concentrated) stock of quinine was prepared and used in all further experimentation

A 500µg/ml (25 µg/ml , 20x concentrated) stock solution of CRDS was prepared and used in all further experimentation

(ii) Generation of dose response curves and construction of the isobologram

The above stock solutions were prepared as described previously (section 2.2.2) and the two drugs were mixed in fixed ratios (Berenbaum, 1978). The respective dose-response curves and IC_{50}'s for each drug ratio were determined from serial dilutions carried out in triplicate using Enzfitter Software®. Parasite growth over a double cycle was assessed using the hypoxanthine method (section 2.2.4). An isobologram was then constructed (details according to section 6.4.2) and the results analysed.

6.3.2.2 CRDS in combination with chloroquine on the *P. falciparum* 3D7 strain

(i) Preparation of the chloroquine and CRDS stock solutions

A 250nM (20x concentrated) stock of chloroquine, and a 500µg/ml (25 µg/ml , 20x concentrated) stock of CRDS was prepared .

(ii) Construction of dose-response curves and isobologram for this combination experiment

The methodology for creating dose-response curves and an isobologram for this combination experiment was as for 6.3.2.1 above.

6.3.2.3 CRDS in combination with mefloquine on the *P. falciparum* 3D7 strain

(i) Preparation of the mefloquine and CRDS stock solutions

A 400nM (20x concentrated) stock of mefloquine and a 500µg/ml (25 µg/ml , 20x concentrated) stock of CRDS was prepared for this combiantion.

(ii) Construction of dose-response curves and and isobologram for this combination experiment

The methodology for creating dose-response curves and an isobologram for this combination experiment was as for 6.3.2.1 above.

6.3.2.4 CRDS in combination with quinine on the *P. falciparum* FCR-3 strain

(i) Preparation of the quinine and CRDS stock solutions

A 2.5µM (20x concentrated) stock of quinine and a 500µg/ml (25 µg/ml , 20x concentrated) stock of CRDS was prepared.

ii) Construction of dose-response curves and and isobologram for this combination experiment

The methodology for creating dose-response curves and an isobologram for this combination experiment was as for 6.3.2.1 above.

6.3.2.5 CRDS in combination with chloroquine on the *P. falciparum* FCR-3 strain

(i) Preparation of the chloroquine and CRDS stock solutions

A 1.25μM (20x concentrated) stock of chloroquine and a 500μg/ml (25 μg/ml , 20x concentrated) stock of CRDS was prepared.

(ii) Construction of dose-response curves and and isobologram for this combination experiment

The methodology for creating dose-response curves and an isobologram for this combination experiment was as for 6.3.2.1 above.

6.3.2.6 CRDS in combination with mefloquine on the *P. falciparum* FCR-3 strain

(i) Preparation of the mefloquine stock solution

A 250nM (20x concentrated) stock of mefloquine and a 500μg/ml (25 μg/ml , 20x concentrated) stock of CRDS was prepared.

(ii) Construction of dose-response curves and and isobologram for this combination experiment

The methodology for creating dose-response curves and an isobologram for this combination experiment was as for 6.3.2.1 above.

6.4 RESULTS

6.4.1 THE EFFECT OF CURDLAN SULPHATE ON *P. FALCIPARUM* OVER A SINGLE AND DOUBLE CYCLE OF GROWTH

With reference to Figure 6.1, there was no effect on *P. falciparum* over a single cycle up to a concentration of 3000 μg/ml. The effect on the parasite could only be observed in the double cycle experiment. It is proposed that curdlan blocks merozoite invasion. Consequently , it only exhibits antimalarial activity in the second cycle of the hypoxanthine assay. To overcome this, the plates were incubated for 78 hours.

The mean IC_{50} derived from three independant experiments was 4.45 ± 1.12 (standard error) μg/ml.

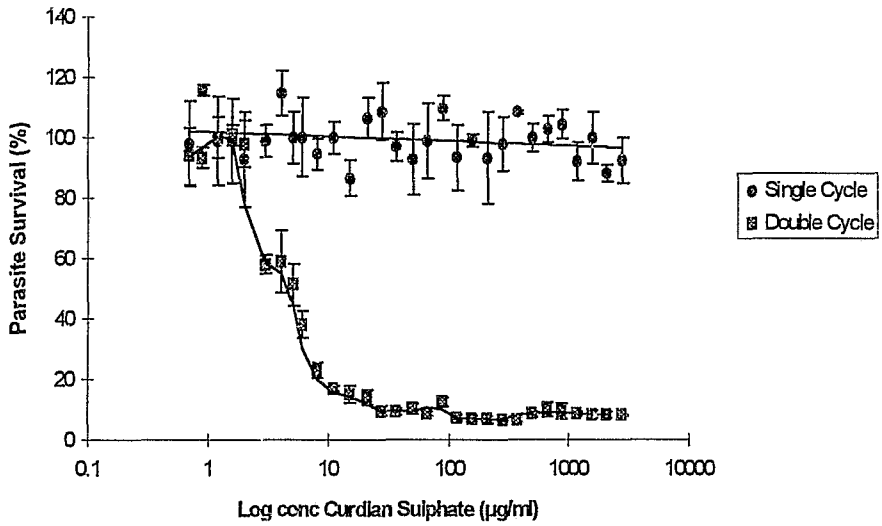

Figure 6.1: The effect of curdlan sulphate on *P. falciparum* (strain FCR3) over one (42 hour) and two (78 hour) schizogony cycles *in vitro*. Parasite growth is expressed as a percentage of growth in a control, untreated culture determined by [^{3}H] incorporation. Each point is a mean(± standard deviation) of at least 3 experiments, using synchronous ring stage cultures with 0.5% parasitaemia and 1% haematocrit.

6.4.2 THE EFFECT OF CRDS IN COMBINATION WITH QUININE, CHLOROQUINE AND MEFLOQUINE ON THE FCR3 AND 3D7 STRAINS OF *P. FALCIPARUM*

The procedure for deriving an isobologram is demonstrated below.

Figures 6.2 to 6.5 show the dose response curves generated for different ratios of quinine in the presence curdlan sulphate against the sensitive 3D7 strain. The percent parasite survival is indicated on the vertical axis (effect) and the log of the quinine concentrations (log C) are represented on the horizontal axis.

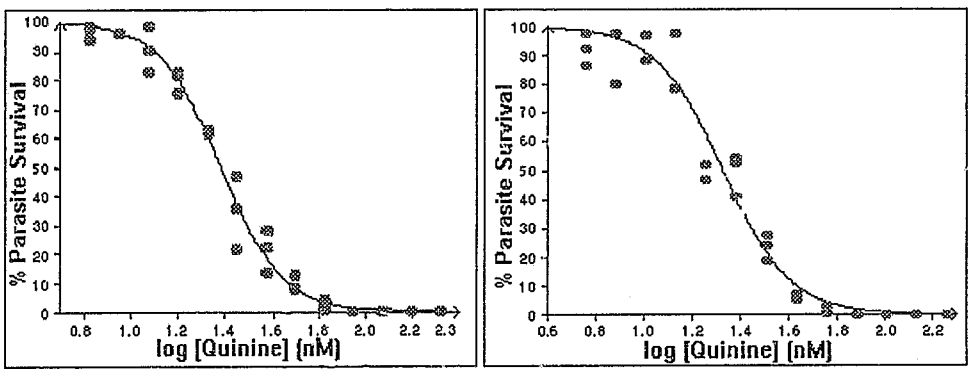

Figure 6.2: The dose response curves generated for plates A and B. Plate A (left) is 280nM quinine and 5µg/ml CRDS in combination, with an IC_{50} value of 24.1 nM. Plate B (right) is 240 nM QN and 7.5 µg/ml CRDS in combination, with an IC_{50} value of 21.2 nM.

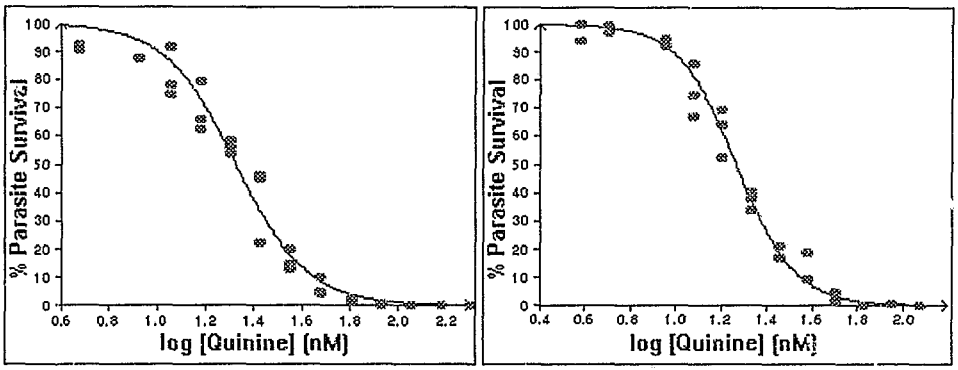

Figure 6.3: The dose response curves generated for plates C and D. Plate C (left) is 200nM quinine and 10μg/ml CRDS in combination, with an IC_{50} value of 21.2 nM. Plate D (right) is 160 nM quinine and 12.5 μg/ml CRDS in combination, with an IC_{50} value of 18.5 nM.

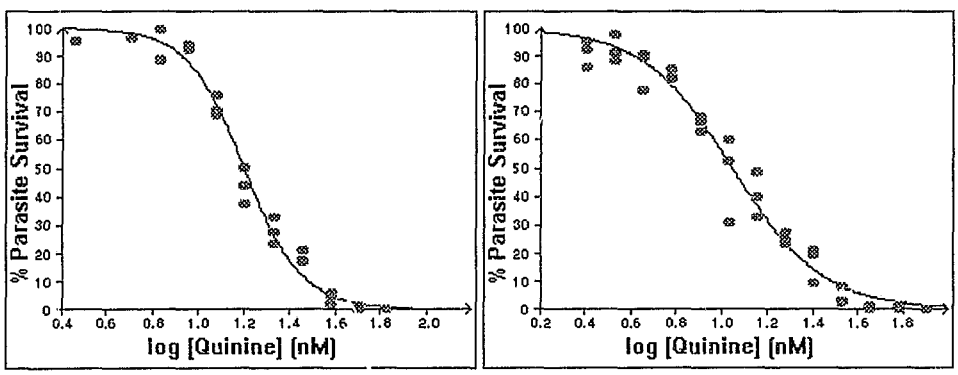

Figure 6.4:The dose response curves generated for plates E and F. Plate E (left) is 120nM quinine and 15μg/ml CRDS in combination, with an IC_{50} value of 15.9 nM. Plate F (right) is 80 nM quinine and 20 μg/ml CRDS in combination, with an IC_{50} value of 11.1 nM.

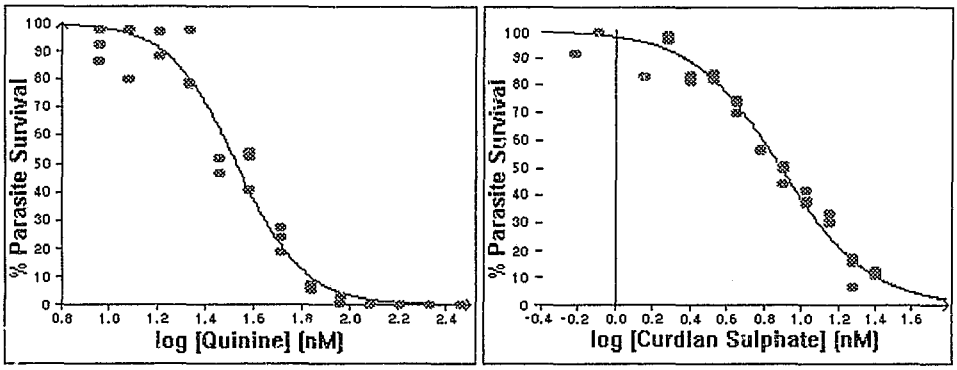

Figure 6.5: The dose response curves generated for plates G and H. Plate G (left) is 400nM quinine plated out alone, with an IC_{50} value of 30.6nM. Plate H (right) is 25 µg/ml CRDS alone, with an IC_{50} value of 7.75µg/ml.

From these eight dose response curves, eight IC_{50} values for the quinine were calculated (Table 6.2). In turn, the IC_{50} values of the curdlan sulphate were calculated using direct proportion. The drug ratio values were calculated by assigning the IC_{50} values of plates G and H equal to the value of one. Then by using direct proportion, the other drug ratio values were calculated. These drug ratio values were then used to construct the resulting isobologram (Figure 6.7), from which the nature of the interaction between the two drugs was then determined. Figure 6.6 is a representation of a typical isobologram indicating the different drug interactions resulting from combination experiments.

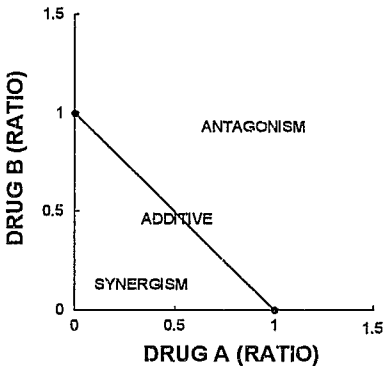

Figure 6.6: A representation of a typical isobologram indicating different drug interactions.

The superimposed solid line (zero interactive line) indicates the locus of where the points would have been , had there been no interaction between the two drugs in combination Points low the zero interactive line, indicate synergism. Points above the zero interactive line indicate an antagonistic interaction between the two drugs. Whilst any points on or in the very near vicinity of the zero interactive line indicate an additive interaction.

Table 6.1: Data used to generate isobolograms. Fixed ratios of drugs used in the combination experiments and the IC_{50} values obtained from Figure 6.2, to generate the isobologram, in Figure 6.3. Where QN is quinine and CRDS is curdlan sulphate.

PLATE	CONCENTRATION		IC_{50} VALUE		DRUG RATIO	
	QN (nM)	CRDS (μg/ml)	QN (nM)	CRDS (μg/ml)	QN	CRDS
A	280	5	24.1	0.4304	0.7876	0.0555
B	240	7.5	21.2	0.6625	0.6926	0.0855
C	200	10	21.2	1.0600	0.6928	0.1368
D	160	12.5	18.5	1.4453	0.6046	0.1865
E	120	15	15.9	1.9875	0.5196	0.2565
F	80	20	11.1	2.7750	0.3627	0.3581
G	400	0	30.6	0	1	0
H	0	25	0	7.7500	0	1

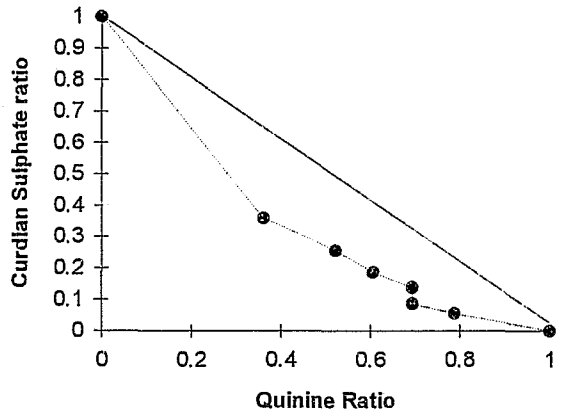

Figure 6.7: Isobologram depicting the interaction of curdlan sulphate with quinine against a chloroquine sensitive strain (3D7) of *Plasmodium falciparum*

Since the points in Figure 6.7 lie below the line, the combination of Curdlan Sulphate and Quinine was found to be synergistic in the *P. falciparum* FCR-3 strain.

Figures 6.8 - 6.12 show the isoboles representing the interaction of curdlan sulphate with different quinolone antimalarials on the chloroquine-resistant (FCR-3) and chloroquine-sensitive(3D7) strains of *P. falciparum.* The combination dose response curves for these combinations are not shown, but the isoboles were constructed in the same manner as indicated for the quinine/curdlan sulphate combination above. Experiments were performed in triplicate. The combinations of curdlan sulphate with chloroquine and mefloquine in the chloroquine-sensitive 3D7 strain and with quinine, chloroquine and mefloquine in the chloroquine resistant FCR_3 strain, were all synergistic.

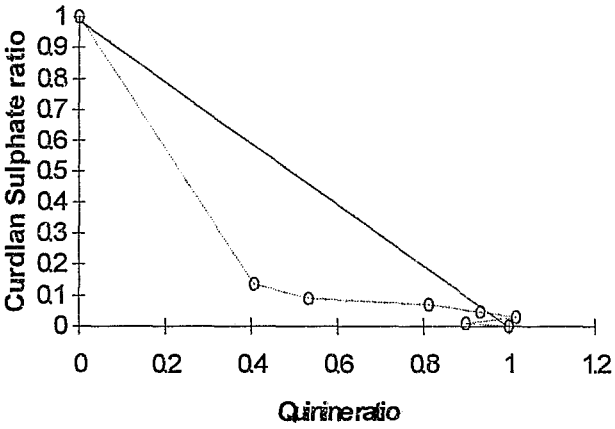

Figure 6.8: Isobologram depicting the interaction of curdlan sulphate with quinine against the chloroquine resistant (FCR3 strain of *P. falciparum in vitro*

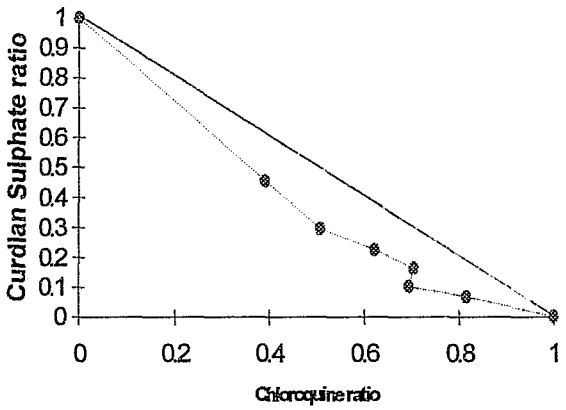

Figure 6.9: Isobologram depicting the interaction of curdlan sulphate with chloroquine against the chloroquine sensitive (3D7) strain of *P. falciparum in vitro.*

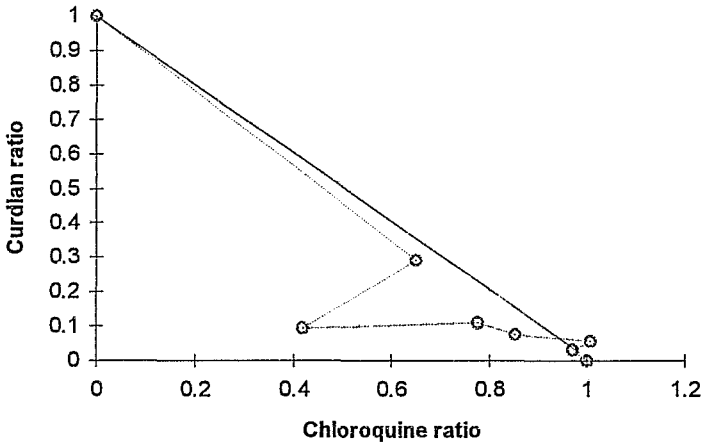

Figure 6.10: Isobologram depicting the interaction of curdlan sulphate with chloroquine against the chloroquine-resistant (FCR-3) strain of *P. falciparum in vitro.*

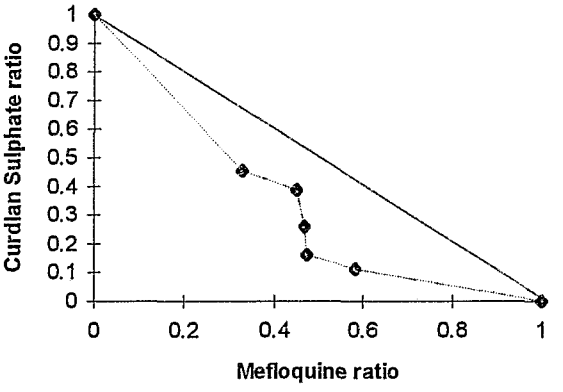

Figure 6.11: Isobologram depicting the interaction of curdlan sulphate with mefloquine against the chloroquine-sensitive (3D7) strain of *P. falciparum in vitro.*

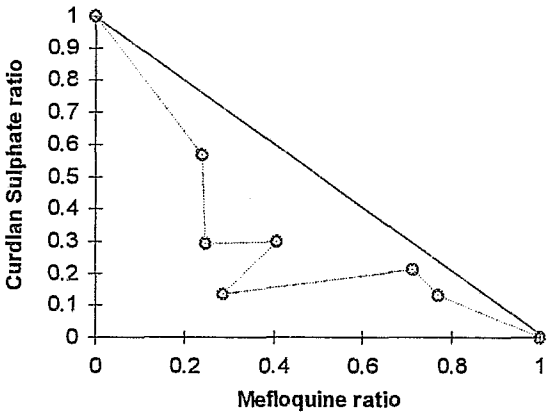

Figure 6.12: Isobologram depicting the interaction of curdlan sulphate with mefloquine against the chloroquine-resistant (FCR-3) strain of *P. falciparum in vitro.*

6.5 DISCUSSION

The anti-HIV activity of CRDS is closely related to the compound's molecular weight and sulphur content. It has been found that CRDS should have a molecular weight greater than 5.0×10^4 daltons and contain greater than 13.5% sulphur in order to possess inhibitory action on HIV-1 infection at a CRDS concentration of 3.3 μg/ml (Kaneko *et al.*, 1989). The CRDS supplied to us by the Ajinomoto Co. for use in these experiments, had an average molecular weight of 6.5×10^4 daltons, and a sulphur content of 14.0%.

With reference to Figure 6.1, curdlan sulphate exhibited no effect on *P. falciparum* over a single cycle of growth. The effect on the parasite could only be observed in the double cycle experiment, where an IC_{50} of 4.45 ± 1.12 μg/ml was obtained. These results support the hypothesis that CRDS interferes with merozoite invasion (Evans *et al.*, 1998). The mechanism by which merozoite

attachment to the red blood cell wall is prevented, is most probably similar to the mechanism by which adsorption of HIV-1 virions to target cell surfaces is prevented (Kaneko *et al.*, 1990). Presumably, CRDS binds to the protein stuctures of merozoites (and possibly erythrocytes), thereby forming a mechanical barrier which prevents their contact with, and subsequent invasion of, red blood cell membranes.

As observed in Figure 6.1, this blocking effect was not complete. Approximately 8% of the parasites still grew even at the highest concentration of CRDS. The exact mechanism for this is not clear. Since this effect was observed in repeated experiments, further experiments were then carried out on CRDS in combination with the classical antimalarials - chloroquine, quinine and mefloquine.

The combination experiments with CRDS and the aminoquinolones chloroquine, quinine and mefloquine showed synergistic effects when tested on both the chloroquine sensitive (3D7) and chloroquine resistant (FCR3) strains of *P. falciparum* (Figures 6.7 to 6.12).

6.6 CONCLUSIONS

* Curdlan sulphate has no inhibitory effect on *Plasmodium falciparum* over a single cycle of growth *in vitro.*
* The IC_{50} for curdlan sulphate over a double cycle of *P. falciparum* growth *in vitro* is 4.45 ± 1.12 μg/ml.
* Combinations of curdlan sulphate with the aminoquinolones quinine, chloroquine and mefloquine against the chloroquine-sensitive (3D7) and chloroquine-resistant (FCR3) strains of *P. falciparum*, are all synergistic.

7. CHAPTER SEVEN - *IN VIVO* MICE EXPERIMENTATION WITH CURDLAN SULPHATE AND *PLASMODIUM BERGHEI*

7.1 INTRODUCTION

The successful results obtained in *in vitro* experimentation (CHAPTER 6) with curdlan sulphate, prompted further *in vivo* investigation using this compound. Preliminary toxicity studies in animal models, have highlighted the low toxicity of this compound at extremely high doses (Kaneko *et al.*,1990). It has also been shown in Phase I/II trials in HIV patients in the USA, that Curdlan Sulphate produced dose-related increases in CD4 lymphocytes in HIV-infected patients after a 4 hour infusion and no clinical side effects were observed at any dose tested (Yoshida T, *et al.*1995). Since CRDS has been used successfully in human HIV patients, if studies with CRDS in malaria *in vivo* yielded successful results, this would in turn prompt the use of this compound in human malaria patients.

7.2 AIMS

* To determine the effect of curdlan sulphate on the clinical course and outcome of malaria infection in mice.
* To develop a dose response curve using a wide range of curdlan sulphate concentrations in *Plasmodium berghei* infected mice.

7.3 METHODOLOGY

The following animal ethics clearance numbers were obtained from the Central Animal Services Unit at the University of the Witwatersrand for this section of experimentation: **92/104/4; 94/16/4**

7.3.1 PILOT EXPERIMENTATION WITH CURDLAN SULPHATE

The following procedure was followed for all the pilot experimentation with the CRDS:

On Day 0, both the control and experimental animals were infected i.p. with 0.1ml *P.berghei* inoculum (administration of 10^6 parasitised erythrocytes). Parasitaemias of > 1% were the indication for starting treatment of the experimental animals with the various doses of CRDS indicated below. The animals' weights and parasitaemias were monitored daily (as per sections 2.4.2.1 and 2.4.2.2). The animals were euthanased when either (i) their body mass had dropped by 15% or (ii) their parasitaemias had reached ± 50%. The animals were then anaesthetised and bled from the tail vein in order to obtain blood for haematocrit calculation (as per section 2.4.1.5). The animals were then euthanased and their spleens surgically removed and weighed in order to calculate spleen indices (as per section 2.4.1.6).

7.3.1.1 Curdlan sulphate preparation

The CRDS was donated by the Ajinomoto Co., Inc., Tokyo, Japan. It was prepared for dilution in sterile phosphate buffered saline (PBS) to give the dose required for 10g of mouse body weight in 0.1ml.

7.3.1.2 Development of experimental inoculum of *Plasmodium berghei*

The procedure for inoculum development was carried out as per section 2.4.1.3.

7.3.1.3 Experimentation with curdlan sulphate at 10mg/kg/day (i.v), 6 hourly

Number of mice required: 15 Female Balb/c mice

The 15 mice were divided into 3 groups - 2 Experimental and 1 Control. The experimental and control mice were inoculated with *P. berghei* on Day 0. From Day 2, treatment of the 10 experimental animals with Curdlan Sulphate commenced. The experimental animals were administered 10mg/kg CRDS into the tail vein, on a 6 hourly basis. The control animals were administered a saline placebo i.v. into the tail vein, 6 hourly. The last dose of CRDS was administered on Day 5. Three experimental animals died during the night. The remaining animals were euthanased on Day 6.

7.3.1.4 The effect of curdlan sulphate at 100 mg/kg/day (i.v and i.p), 6 hourly

Number of animals required: 15 Female Balb/c mice

The 15 mice were divided into 3 groups - 2 Experimental and 1 Control. The experimental and control mice were inoculated with *P. berghei* on Day 0. From Day 3, treatment of the 10 experimental animals with curdlan sulphate. The experimental animals were administered 100mg/kg CRDS into the tail vein, on a 6 hourly basis. The control animals were administered a saline placebo i.v. into the tail vein, 6 hourly. Initially, the animals were administered the drug or placebo i.v., but later due to difficulties with this method of administration (the tail veins became bruised/damaged), the drug or placebo had to be administered i.p. The animals' weights and parasitaemias were monitored daily. The last dose of CRDS was administered on Day 6. The animals were euthanased on Day 7 (the "end day").

7.3.1.5 Experimentation with curdlan sulphate at 200mg/kg/day (s.c.), 6 hourly

Number of animals required: 10 Female Balb/c mice

The 10 mice were divided into 3 groups - 2 Experimental (8 mice) and 1 Control (2 mice). The experimental and control mice were inoculated with *P. berghei* on Day 0. From Day 3, treatment of the 10 experimental animals with Curdlan Sulphate commenced. The experimental animals were administered 200mg/kg CRDS subcutaneously, on a 6 hourly basis. The control animals were administered PBS, subcutaneously, 6 hourly. The last dose of CRDS was administered early on Day 6. The animals were euthanased later on Day 6 (the "end day").

7.3.2 DEVELOPMENT OF A DOSE-RESPONSE CURVE FOR CURDLAN SULPHATE IN *P.BERGHEI* INFECTED MICE

Since the doses of CRDS used in previous experimentation were extremely high, this section of experimentation was carried out in order to determine the minimal effective drug dose necessary to inhibit the parasite *in vivo*. i.e to develop a dose response curve for CRDS in *P.berghei* infected mice.

The experimental procedure was carried out in two stages in order to facilitate easier handling of the mice. 52 Inbred female Balb/c mice were used : 12 for the development of the *P.berghei* inoculum to be used in the experimental groups (6 in each stage) and 20 for each of the two experimental stages. The 20 experimental mice were randomly assigned, 5 per cage for treatment with different doses of CRDS.

The treatment groups were as follows:

(i) Stage 1 :

0 mg/kg CRDS (control), 200 mg/kg CRDS, 100 mg/kg CRDS, 50 mg/kg CRDS

(ii) Stage II :

0 mg/kg CRDS (control), 25 mg/kg CRDS, 12.5 mg/kg CRDS, 5 mg/kg CRDS

7.3.2.1 Stage 1

(a) Development of experimental inoculum of *P.berghei*

As per section 2.4.1.3

(b) Experimental procedure

Immediately after the development of the experimental inoculum, the mice in the treated groups were each infected intraperitoneally with 0.1ml inoculum (administration of 10^6 parasitised erythrocytes). The mice weights and parsitaemias were recorded on a daily basis. When the parasitaemia reached ~ 10%, the mice began receiving a particular dose of CRDS. The drug was injected subcutaneously at 6 hour intervals.

Group 1 was administered 0 mg/kg (control group) CRDS/day, Group 2 : 200mg/kg CRDS/day, Group 3: 100mg/kg CRDS/day. On the eighth day, or when the parasitaemia approached 50%, the mice were euthenased. Haematocrits and spleen indices were then determined as previously described.

7.3.2.2 Stage 2

(a) Development of experimental inoculum of *P.berghei*

As per section 2.4.1.3.

(b) Experimental procedure

Stage 2 was carried out as described in Stage 1, but three lower doses of drug were used in this experiment. Group 1 was administered 0mg/kg CRDS/day (control group), Group 2: 25mg/kg CRDS/day, Group 3: 12.5 mg/kg CRDS/day and Group 4: 5mg/kg CRDS/day.

7.4 STATISTICAL ANALYSIS

The results of the parasitaemia were expressed as the Mean ± SD. The statistical significance of the difference between the control and the CRDS treated groups was evaluated using the student's *t* test. The difference was considered significant when $P < 0.05$.

7.5 RESULTS

7.5.1 THE EFFECT OF CURDLAN SULPHATE AT 10MG/KG/DAY I.V. ON *P.BERGHEI* INFECTION IN MICE

Curdlan sulphate at 10mg/kg i.v had no effect on the mean parasiteamias, spleen indices, haematocrits or weight loss in the experimental group compared to the control (Table 7.1).

Table 7.1: Mean ± SD of "end day" Parasitaemias, Spleen Indices, Haematocrits and Weight Loss.

Group	"End Day" (Day 6) Parasitaemias	Spleen Indices	Haematocrits (%)	Weight Loss (g)
Experimental	40.03 ± 5.49	1.57 ± 0.22	28.81 ± 2.57	1.96 ±0.70
Control	42.83 ± 12.26	1.60 ± 0.23	29.22 ± 3.68	1.74 ± 0.81

7.5.2 THE EFFECT OF CURDLAN SULPHATE AT 100MG/KG/DAY (I.V AND I.P)

Administration of CRDS at 100 mg/kg/day resulted in very little difference in mean spleen indices or weight loss between the control and experimental groups, and a mean haematocrit about 25% higher in the experimental group (Table 7.2) compared to the control group. However, a dramatic difference was observed in "end day" parasitaemias - 9.28% in the experimental group compared to 60.7 % in the control group (Figure 7.1).

Table 7.2: Mean ± SD of "end day" Spleen Indices, Haematocrits and Weight Loss for the experimental and control mice

Group	Spleen Indices	Haematocrit (%)	Weight Loss (grams)
Experimental	2.06 ± 0.20	33.77 ± 6.14	1.47 ± 0.99
Control	1.98 ± 0.26	25.92 ± 0.97	1.56 ± 1.18

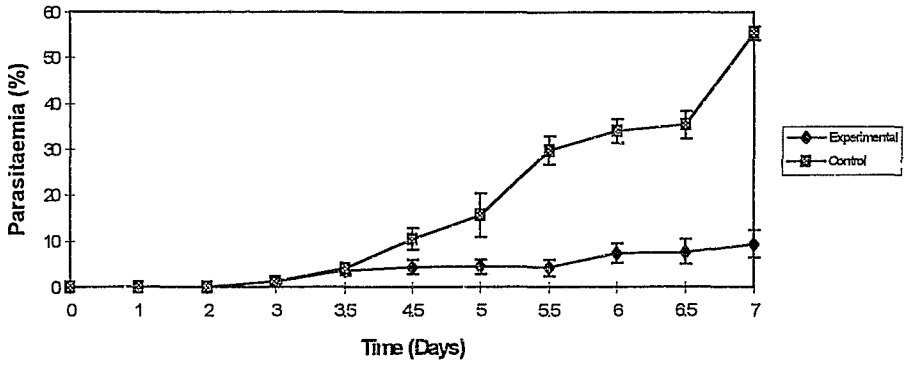

Figure 7.1: Mean ± SD of Daily Parasitaemias for the experimental and control mice

7.5.3 THE EFFECT OF CURDLAN SULPHATE AT 200MG/KG/DAY (S.C), 6 HOURLY

Curdlan sulphate administered at 200mg/kg/day subcutaneously, resulted in a higher mean spleen index and mean haematocrit which was approximately 40% higher in the experimental group compared to the control (Table 7.3). Weight loss was lower in the experimental group (Table 7.3). With reference to "end day" parasitaemias (Figure 7.2), however, there was once again a dramatic difference between the experimental (4.97%) and control (54.5%) groups.

Table 7.3: Mean ± S.D of "end day" Spleen Indices, Haematocrits and Weight Loss for the .perimental and control animals

Group	Spleen Indices	Haematocrit (%)	Weight Loss (g)
Experimental	1.76 ± 0.14	33.80 ± 8.44	1.04 ± 0.40
Control	1.25 ± 0.29	20.00 ± 4.24	1.53 ± 0.31

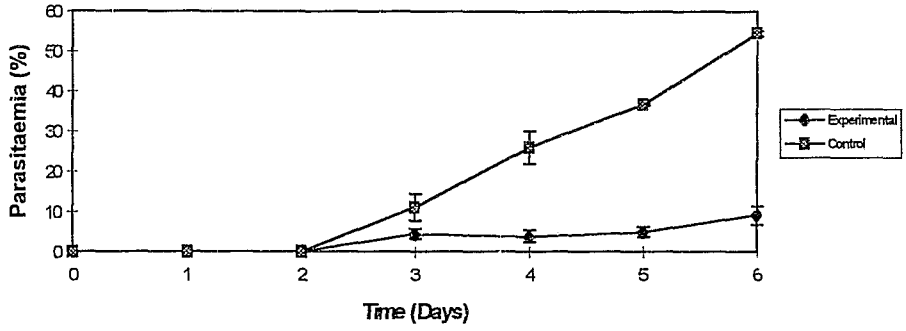

Figure 7.2: Mean ± SD of daily parasitaemias for the experimental and control mice

7.5.4 DEVELOPMENT OF DOSE-RESPONSE CURVES FOR CURDLAN SULPHATE IN *P.BERGHEI* INFECTED MICE

Figures 7.3 and 7.4 respectively show the effect of CRDS on parasite growth for experimental Stages 1 and 2. The effectiveness of the CRDS was measured via its ability to inhibit the course of parasitaemia in the mice. In the control (untreated) mice , there was an exponential increase in parasitaemia until the parasitaemia approached 50% (at which time the mice were euthanased). In the treatment groups, the rate of increase of parasitaemia was inhibited in a dose-dependent manner until an increase in parasitaemia was achieved (Figure 7.5).

As far as the effect of the various CRDS doses on parasitaemia, is concerned, there was no significant difference in the efficacy of CRDS in the groups receiving doses 50mg/kg/day, 100mg/kg/day and 200 mg/kg/day. The experimental groups receiving the lower doses of CRDS, demonstrated a decrease in efficacy of the compound with a corresponding decrease in dose (Figure 7.5).

The estimated IC_{50} value for CRDS *in vivo* is 25mg/kg/day. This value was derived from Figure 7.5 which shows the dose-dependant effect of CRDS on the fractional rate of change in pasitaemia/day.

Table 7.6 shows that the mean "end day" haematocrits obtained from the animals administered the two higher doses of CRDS (200 and 100mg/kg/day), were significantly lower than the haematocrits obtained for either the other treated animals (CRDS doses of 5, 12.5 and 25 mg/kg/day) or the control groups .Also, the spleen indices at the varying doses of CRDS in treated and untreated groups were not significantly different.

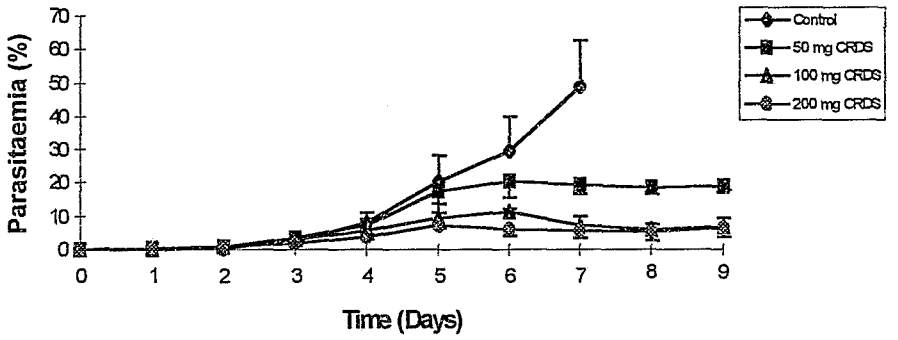

Figure 7.3: Dose-response curve showing the effect of 0, 50, 100 and 200 mg CRDS/kg/day on parasitaemia in Balb/c mice (Stage 1)

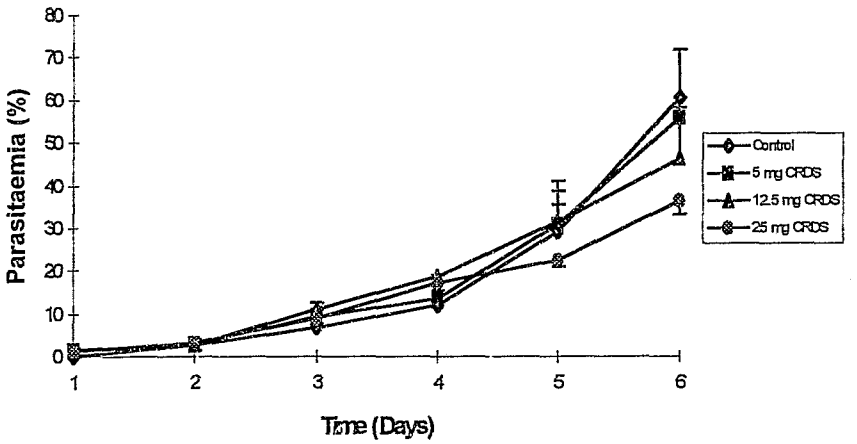

Figure 7.4: Dose-response curve showing the effect of 0, 5, 12.5 and 25 mg CRDS/kg/day on parasitaemia in Balb/c mice (Stage 2)

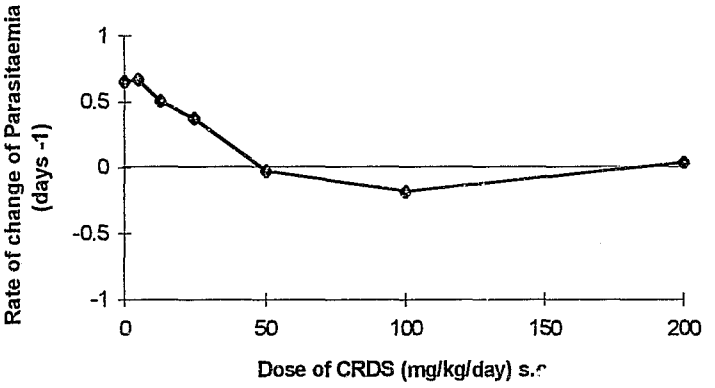

Figure 7.5: The dose-dependent effect of CRDS on the fractional rate of change in parasitaemia per day.

Table 7.4: The effect of varying concentrations of CRDS on the haematocrits and spleen indices in Balb/c mice.

Dose of CRDS	**"End day" haematocrit (%)** (Mean ± SD)	**Spleen Indices** (Mean ± SD)
PART A		
Control	34.5 ± 4.9	1.22 ± 0.07
200 mg/kg/day	22.0 ± 1.4	1.83 ± 0.15
100 mg/kg/day	26.3 ± 1.3	1.87 ± 0.10
50 mg/kg/day	31.0 ± 2.6	1.74 ± 0.11
PART B		
Control	22.8 ± 0.7	1.21 ± 0.06
25 mg/kg/day	25.9 ± 8.2	1.19 ± 0.05
12.5 mg/kg/day	21.3 ± 1.4	1.22 ± 0.03
5 mg/kg/day	22.9 ± 1.3	1.42 ± 0.50

7.6 DISCUSSION

Using the recommended dose of curdlan sulphate of 10mg/kg/day i.v., 6 hourly (the equivalent effective *in vitro* level of 3 μg/ml), had no effect on the outcome of malaria infection in the experimental group compared to the control group (with reference to Table 7.1, there was no difference between the two groups as far as "end day" parasitaemias, spleen indices, haematocrits or weight loss is concerned).

Since difficulties were experienced with the i.v. method of administration, the animals may have received an insufficient dose of drug to begin with (the drug may have been given extravascularly thus producing lower peak levels compared to i.v. administration).

Also, since curdlan sulphate is administered in man via continuous infusion ($T_{½}$ = 60 minutes), administering the drug 4 hourly i.v. in the mice could have resulted in intermittent dropping of the drug concentration in the blood below the therapeutic level. Considering that this drug is effective *in vitro* (refer to experimentation in CHAPTER 6), it was essential to repeat the experimentation, using a higher dose of drug.

When the curdlan sulphate was administered at 100mg/kg/day (i.v + i.p), 6 hourly (Table 7.2), slight differences between the control and experimental groups were observed as far as mean spleen indices and weight loss were concerned. The mean haematocrit observed for the experimental animals was higher than that of the controls. In Figure 7.1, however, a dramatic difference was evident between the control and experimental groups as far as "end day" parasitaemia was concerned (9% for the treated animals compared to 55% for the controls). Although the CRDS did not clear the parasitaemia, it definitely slowed down parasite multiplication.

The greatest problem encountered in this experiment, was difficulty with the injection of the drug and placebo. Initially, the curdlan sulphate and the PBS were administered via the tail vein.

Eventually, the tail veins became damaged and were very difficult to find, so the animals were injected intraperitoneally. Difficulties with this method of injection were also encountered. Some mice died due to injuries caused by i.p. administration (haematomas and G.I.T) injuries. Even if the injuries were not lethal, monitored values such as haematocrit and weight could have been affected. Since there was no data available on CRDS pharmacokinetics after subcutaneous drug administration, the above experiment was repeated using this method for drug and placebo administration in order to verify the results obtained.

Subcutaneous administrationof the curdlan sulphate at 200mg/kg/day (s.c), 6 hourly, resulted in a higher mean spleen index, and a higher mean haematocrit in the experimental group compared to the control group (Table 7.3). The higher haematocrit value may have indicated a protective effect of CRDS on the mice, but difficulties with the haematocrit equipment may have influenced the obtained value. Weight loss was slightly lower in the experimental group compared to the control (Table 7.3). In Figure 7.2, a dramatic difference in "end day" parasitaemias can once again be noted between the curdlan sulphate treated and control animals (5% for the treated animals compared to 55% in the control animals).
Since the subcutaneous method of administration was quick, easy and very successful it was the chosen route of administration for further experimentation.

In the final set of experiments with *Plasmodium berghei* infected mice, a dose-response curve for curdlan sulphate was determined .

The results of this study demonstrate that CRDS inhibited parasite growth *in vivo*. Figure 7.3 shows that the parasitaemia increased marginally on the first day of CRDS administration, but this was

followed by a zero rate of parasite growth on subsequent days of treatment. This indicates that the compound has a static effect on parasite growth. The initial increase in parasitaemia is assumed to be due to the fact that CRDS's effect is only seen over a double cycle of growth i.e. at the time of reinvasion (Havlik *et al.*, 1994). This further supports the presumption that CRDS interferes with merozoite reinvasion.

Erythrocyte invasion by the merozoite is a process essential to the survival of malaria parasites (Hadley and Miller, 1988). Proteins on the surface of these merozoites are thought to mediate initial recognition and attachment to the red blood cell and it has been proposed that the merozoite surface protein -1 is a ligand for red blood cell binding (Perkins, 1989). The CRDS possibly coats the merozoites and prevents the attachment of the surface proteins (msp 1) to the receptors of the target cell. thereby preventing the merozoites from invading the red blood cells. However, the exact mechanism of this blocking effect is not clear, since the prevention of merozoite invasion is not complete - as observed in this experiment and in previous *in vitro* experiments (Havlik *et al.*, 1994).

Table 7.6 shows that the mean "end day" haematocrits of the experimental animals administered the two highest doses of CRDS were significantly lower than those of the control animals. This effect, in conjunction with the severe bruising observed on the tails from these two groups was probably caused by to the anticoagulant properties of CRDS at higher doses. The anticoagulant activity of CRDS has been determined to be 14 to 16 units/mg , which is approximately one tenth that of heparin ,130 to 150 units/mg (Kaneko *et al.*, 1989). At the reduced doses of CRDS administration , this effect was not observed. . The effects observed with respect to the decrease in haematocrits at CRDS doses of 100 and 200mg/kg/day , have been previously described in chronic toxicity studies (Ajinomoto Co, 1994 - unpublished work) and have been found to be reversible when the treatment is withdrawn.

The toxicity profile of CRDS *in vivo* has been shown by Kaneko *et al.*(1990), to be favourable. In experiments with Sprague-Dawley rats, neither death nor haemorrhage was observed with consecutive administrations of CRDS for 2 weeks at doses of 50mg/kg/day, and the LD_{50} of CRDS injected intravenously in mice or rats was found to be around 2000mg/kg.

CRDS has a half life proportional to its molecular weight : 60 min with M.W. of 7×10^4 daltons and 180 min with 17×10^4 daltons (Kaneko *et al.*, 1989) as determined using a rat model. This short half-life, in combination with subcutaneous administration of the drug (which increased the time required for absorption), resulted in a high IC_{50} value of 25mg/kg/day. The CRDS dosage for continuous infusion is calculated as 6.5-19.5mg/hour or 3.2-9.4 mg/kg/day in humans (Kaneko *et al.*, 1989). It is therefore anticipated that the IC_{50} value of CRDS if administered intravenously, will be lower.

In addition to measurable parameters such as parasitaemia, haematocrit, spleen index and weight loss, the curdlan sulphate treated animals generally showed less severe clinical signs of malaria infection compared to the control animals.

CRDS has been found by Masihi *et al.*(1994), to inhibit tumour necrosis factor alpha (TNF-α) activity. TNF is a cytokine that has been implicated in the severe complications caused by cerebral malaria (Clark, 1989). TNF has been postulated to control malaria parasites at crisis (Clark, 1978), to be triggered by malaria parasites when they undergo schizogony (Bate, 1988) and, along with interleukin-1 and interferon, to mediate the pathology of malaria (Kwiatkowski *et al.*,1990). By inhibiting the production of TNF, CRDS may decrease the exaggerated physiological response to malaria infection.

7.7 CONCLUSION

* CRDS is a new antimalarial compound with possibly a novel mechanism of action. The low toxicity profile, inhibition of parasites *in vitro* and *in vivo*, synergism with the classical antimalarials and the inhibition of TNF, are all characteristics of CRDS that make it a potentially excellent candidate for adjunct therapy with the classical antimalarial drugs.

8. CHAPTER EIGHT - CONCLUDING DISCUSSION

This dissertation has examined the role of two different polysaccharides in malaria. In its non-sulphated form, lentinan was found to exert no effect on the malaria parasite either *in vitro* nor *in vivo*. On the other hand, curdlan in its sulphated form was found to inhibit the malaria parasite both *in vitro* and *in vivo*.

In general, the polysaccharides which have been used as an aid in the management of certain diseases or infections, have the following major properties:

(i) They can exert an immunomodulating effect on their own e.g. lentinan and curdlan

(ii) By sulphating the polysaccharides, their immunomodulating effect can be modified and enhanced ;

(a) In addition to their immunomodulating properties, they can also exert non-specific effects e.g in malaria. The reduction in parasite proliferation by CRDS may either be due to the physical coating of (i) the parasite with subsequent prevention of schizont rupture or (ii) the erythrocyte thereby preventing invasion of the parasite once it has been released from the schizont (Evans *et al.*, 1988).

(b) By the addition of sulphated groups, cytoadherence can be prevented e.g. a study by Pancake *et al.* (1993), indicated that circumsporozoite proteins (the major surface proteins of the sporozoites of the various *Plasmodium* species) bind selectively to certain sulphated glycoconjugates, including heparin. The adherence to and invasion of host hepatocytes by

sporozoites and therefore sporozoite infectivity was subsequently inhibited by this compound.

Polysaccharides, particularly in their sulphated form, therefore play a dual role in their effect. Firstly, they can directly affect proliferation of the parasites in the body and secondly they can act non-specifically by influencing cytoadherence of the parasite to certain host cells.

The cytokine network is assumed to play a crucial role in the host's response to malaria infection. At the time of schizont rupture, macrophages become stimulated by material released from the parasite (malaria toxins). Due to the expression of certain genes, inflammatory cytokines are then triggered and released. Certain cytokines, for example TNF, have been found to play a dual role in both the protection and pathology of malaria (Kremsner, *et al.*,1995). TNF in turn causes the generation of inducible nitric oxide synthase and hence the release of nitric oxide itself. A study carried out by Clark *et al.*(1996), suggest that the extra-neuronal nitric oxide induced by products of schizogony contribute to the seizures and unconsciousness seen in cerebral malaria. The development of novel strategies to block the production of TNF therefore have a potential therapeutic relevance. Masihi *et al.*(1994), have made progress in this regard. They found that significant production of TNF and high oxidative respiratory bursts of phagocytic cells in BCG-primed animals following lipopolysaccharide (LPS) treatment could almost be completely inhibited by either lentinan or Curdlan Sulphate. These results suggest the feasibility of using lentinan and curdlan sulphate as inhibitors of TNF production in clinically relevant conditions, possibly including malaria.

Although these findings suggest that polysaccharides in both their non-sulphated and sulphated forms may play a role in cytokine regulation in various infections, the results from this research

indicate that only polysaccharides in their sulphated form have an effect on the malaria parasite itself. The mechanism by which polysaccharides, and particularly those in their sulphated form, exert this effect in malaria, is not clear. Experiments carried out by Aiakawa *et al.*(1996), have revealed that the membrane knobs of unfixed *Plasmodium falciparum* infected erythrocytes have a positive charge , whereas the remainder of the red cell plasma membrane is negatively charged. Since endothelial plasma membranes have a negative charge, the charge difference between knobs and endothelium may play a significant role in cytoadherence between the two cell types. As far as the interaction between CRDS and various receptors is concerned, the following has been postulated (personal communication): since curdlan in its sulphated form is electronegatively charged (acidic), and receptors are protein structures with both basic and acidic amino acids protruding from the molecule, there is a simple non-specific interaction between the acidic portion of the curdlan molecule and the basic portion of the receptor molecule. In the case of the malaria parasite-infected erythrocyte, the CRDS which is negatively charged, may exert its blocking effect by binding to the positively charged protruding knobs.

When the polysaccharide is in its non-sulphated form, its overall charge is different from that in its sulphated form. Hence, neither the direct blocking effect on the merozoites is seen, nor the effect on cytoadherence. These results are supported by the findings from the experimental work carried out with lentinan.

The section of this dissertation on curdlan sulphate together with several other studies carried out on sulphated polysaccharides, highlights the progress which has been made into determining the mechanism of action of these compounds. However, important avenues of future study with CRDS in parasitology generally and malaria specifically are :

(i) Identification of the combining sites of CRDS to both host cells or *Plasmodium* merozoites using radiolabelled CRDS, and

(ii) The determination of whether the reaction between cellular or parasitological sites and CRDS is specific or nonspecific.

In conclusion, although the exact mechanism of action of CRDS is yet to be elucidated, results obtained from this research study together with preliminary results from clinical trials currently being carried out in human patients (personal communication), indicate that the prospects for using this compound as an adjunct to conventional antimalarials in treating severe and uncomplicated cases of *P.falciparum* malaria, are extremely promising.

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