The use of feeder cells in the cultivation of the asexual erythrocytic stages of *Plasmodium falciparum*

by

K.R. Trenholme B.Sc. (Hons) M.Sc.

Zoology Department

University of Glasgow

A thesis submitted for the degree of Doctor of Philosophy in the University of Glasgow.

December 1987

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

DEDICATION

FOR MUM, JON AND ALAN.

Contents

Chapter	Title	Page
	Acknowledgements	(i)
	Summary	(ii)
1	General Introduction.	1
	Experimental Rationale.	45
2	Materials & Methods.	47
3	Observations on the use of peritoneal wash cells as feeder cells for cultures of *P. falciparum*.	58
4	The adaptation of new isolates of *P. falciparum* to continuous culture using peritoneal wash cells.	92
5	The development of a technique for the cloning of *P. falciparum* (by limiting dilution) using a feeder cell layer of peritoneal wash cells.	103
6	The evaluation of 4 mammalian cell lines as feeder cells for cultures of *P. falciparum*	130
7	The effect of medium preconditioned by peritoneal wash cells, or supplemented with either L-cysteine or 2-mercaptoethanol on the growth of *P. falciparum*	144
8	General discussion	169
	Appendices	
	References	

Acknowledgements

I must thank my supervisor Professor Stephen Phillips for his help and guidance throughout this project. I would also like to express my thanks to Dr Sheila McLean for her invaluable help and moral support.

I am most grateful to Dr Eric Walker and his colleagues at Ruchill Hospital for their help in obtaining isolates of *P. falciparum*. Thanks also to Dr Mary Freshney for supplying the human cell lines Raji, 143 and K562 and to Dr Jana McBride for kindly providing the monoclonal antibodies used in this project.

I am most grateful to Patricia Johnston for typing this thesis so efficiently.

Finally I would like to thank my family for all the help they have given me, and my friends for giving me support and encouragement, and for being there when I needed them most.

This work was undertaken in the Department of Zoology, Glasgow University, between 1984 and 1987 as a research student on an SERC grant (Number B-84318708).

Summary

While the technique of Trager and Jensen (1976) allows the *in vitro* cultivation of the asexual erythrocytic stages of *P. falciparum*, this method does have a number of limitations. Most isolates of *P. falciparum* grow poorly during the first weeks of cultivation and only some new isolates can eventually be established in culture. Methods to increase the proportion of isolates, and also the number of individual parasites within an isolate which could be established in long term culture were studied.

Cultures of *P. falciparum* showed increased multiplication rates in the presence of a feeder cell layer of mouse peritoneal wash cells (PWCS) and within this population the adherent (macrophage enriched) population was the most important in promoting this increase.

New isolates of *P. falciparum* adapted to culture more readily in the presence of a feeder cell layer of PWCS. Thirteen isolates were tested and only 3 were established in culture in the absence of PWCS. A further 6, however were adapted when cultured with PWCS. Three of the 4 isolates which were not established in culture with PWCS were known to have been taken from patients who had been treated with antimalarials and therefore the viability of these parasites at the time of culture was questionable. This was confirmed by the fact that these parasites did not complete a single asexual cycle.

The effect of PWCS in the adaptation of new isolates of *P. falciparum* to long term culture was examined. It was found that

the presence of PWCS led to the preservation of a greater degree of parasite diversity than that which was achieved under standard culture conditions. The presence of a feeder cell layer of mouse PWCS when cloning new isolates of P. falciparum (by limiting dilution) led to greatly increased cloning efficiency. Sixteen clones were produced from isolate AF using this method. These clones were examined using a panel of monoclonal antibodies and three different antigenic types were observed. In an attempt to streamline this cloning technique the feasibility of using cryopreserved PWCS was investigated. This was not, however, successful as the ability of cryopreserved PWCS to promote an increase in parasite growth declined rapidly during storage at -70°C, probably due to a decrease in macrophage viability.

The use of PWCS as feeder cells for cultures of P. falciparum does have a number of disadvantages. It would be simpler and more convenient if an alternative feeder cell which grown continually could be found, dispensing with the need to use mice as a source of PWCS. Three human cell lines (Raji, K562 and 143) were tested as potential feeder cells, but they were unable to promote an increase in parasite multiplication. However a fibroblast like cell line derived from the mountain vole (Microtus montanus) was as effective as PWCS in promoting an increase in parasite multiplication. Medium preconditioned by PWCS and stored at -20°C before use was not effective in promoting an increase in parasite multiplication. Medium preconditioned by PWCS and used immediately promoted some increase in parasite multiplication however. This suggests that a growth and/or viability factor was produced by the PWCS and that this factor was unstable.

An increase in the multiplication rate was also achieved in the absence of feeder cells when cultures of P. falciparum were grown in medium which was supplemented with L-cysteine at 12 hour intervals. This effect was not so marked when medium was supplemented with 2-mercaptoethanol. It was concluded that at least one role of PWCS and M. montanus cells in the promotion of parasite growth and multiplication in vitro is the continuous production of cysteine.

CHAPTER 1

INTRODUCTION

General Introduction

In 1955 the eighth World Health Assembly adopted a policy of malaria eradication. By the early 1970's extensive eradication campaigns had shown considerable successes in Europe, North America, U.S.S.R., Australia, parts of the Near East, Asia and South America. Since 1974, however, the number of reported cases of malaria in parts of S.E. Asia and Mid America has been increasing [WHO 1985].

Interruption of transmission of malaria parasites by house spraying with residual insecticides was the base of the W.H.O. malaria eradication campaign. Initially DDT was widely used for residual house spraying, but as resistance to DDT developed, organophosphates (e.g. Malathion) and carbamates (e.g. Propoxur) were also used. Organophosphate and carbamate insecticides are considerably more expensive than DDT making their use prohibitively expensive for some countries. The effectiveness of residual spraying of houses largely depends on the indoor resting habits of the mosquitoes. It has become increasingly obvious that many malaria vectors especially in Central and South America and South East Asia are exophilic (i.e. rest outside) so that residual house spraying is of little use in reducing malaria transmission. Another problem with house spraying is that it may lead to alterations in vector behaviour. It may kill the endophilic (i.e. indoor resting) portion of the vector population, and in so doing reduce the competition between the larvae of endophilic and exophilic adults leading to an increase in the exophilic and possibly exophagic vector populations.

Thus after a number of years of house spraying, malaria may become a problem again because of a large outdoor biting and resting vector population, as has been the case in parts of S. America. Case diagnosis and drug treatment play a large role in malaria control, but drug resistance of Plasmodium falciparum is expanding rapidly causing difficulties in chemotherapy and chemoprophylaxis.

At the moment P. falciparum is resistant in varying degrees to all available antimalarial drugs e.g. quinine, chloroquine, sulfadoxine and pyrimethamine, and mefloquine. Chloroquine resistance was first reported in Colombia and also Thailand in the late 1950's and is now widespread in Southeast Asia, the Western Pacific, Central and South East India, East and West Africa and South America.

The W.H.O. malaria eradication campaign did not therefore succeed due to administrative and financial problems which were aggravated by the rapid spread of resistance to insecticides, by alterations in vector behaviour and the appearance of drug resistant parasites.

5.5 million cases of malaria were reported in 1983 (excluding Africa south of the Sahara) [W.H.O. 1985]. Malaria remains a major public health problem for a large part of the world's population and places considerable constraints on social and economic development in affected areas.

It is now apparent that present control methods are not only prohibitively expensive but in many areas of the world are no

longer adequate. There is an urgent need for new control measures and a greater understanding of all aspects of malaria infections and parasites will eventually contribute towards this.

The Discovery of Malaria Parasites

In 1880 Laveran first described the crescent shaped bodies we now know to be gametocytes of Plasmodium falciparum in the blood of a malaria patient and these findings were subsequently confirmed by Marchiafava and Celli (1883). Ross demonstrated the mosquito transmission of bird malaria in 1898 [Manson 1898] and this was confirmed for human malaria by Grassi in the same year (reviewed by Harrison 1978). It was not until 1948 when Shortt and Garnham described exoerythrocytic schizonts in the livers of infected monkeys and subsequently in humans [Shortt and Garnham 1948 , Shortt et al. 1948, 1951] that the basic life cycle of the malaria parasite was elucidated.

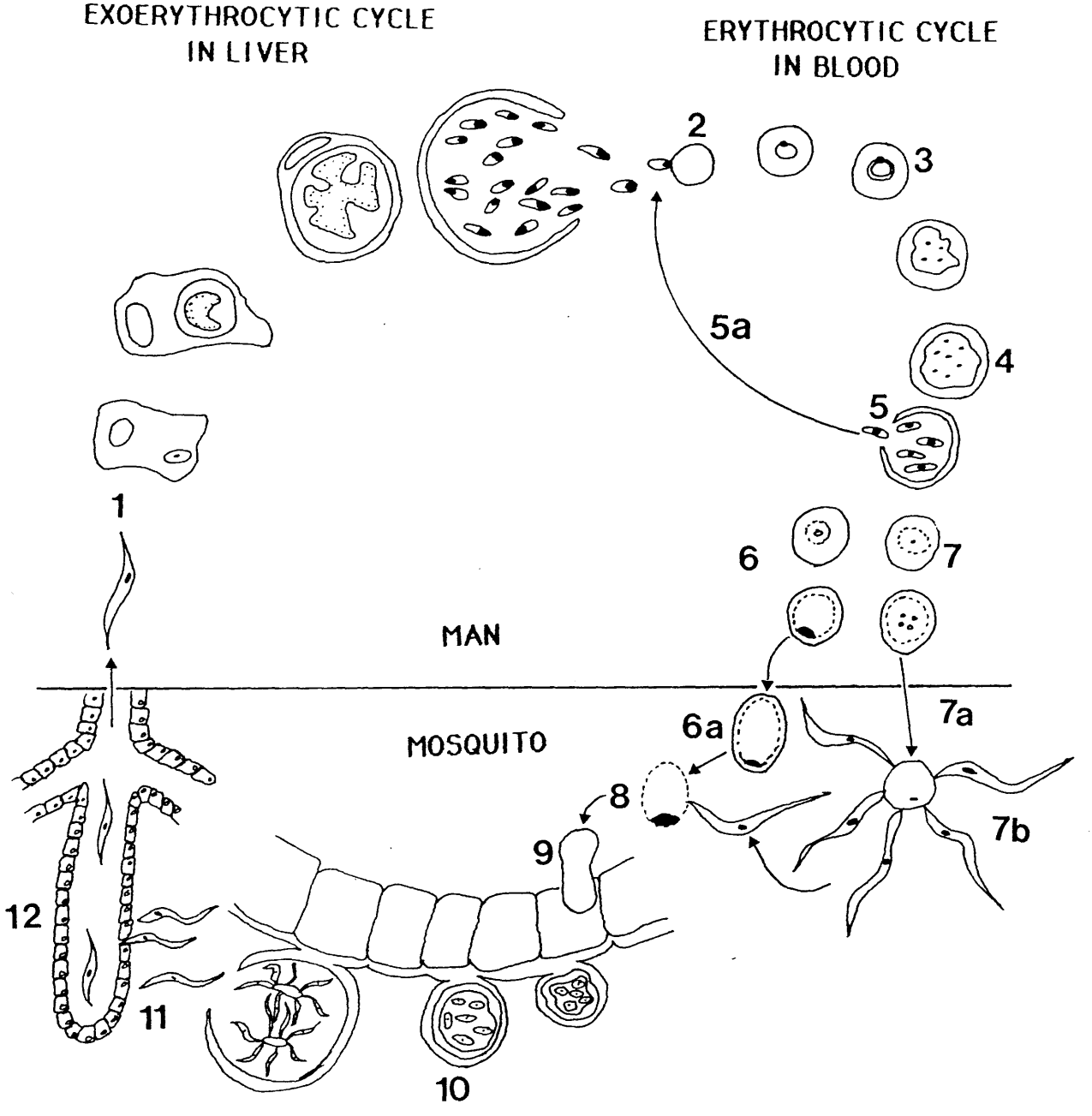
Over 100 species of Plasmodium have now been described in a wide variety of vertebrate hosts such as rodents, primates, birds and reptiles. Four species of Plasmodium normally cause malaria in man, Plasmodium vivax, P. ovale, P. malariae and P. falciparum, the last being the most life threatening infection.

Life Cycle

The life cycle of human malaria parasites as known at the present time is described below, and illustrated in Figure 1.

The infection begins when an infected female anopheline mosquito bites a suitable host. Sporozoites are injected into the

FIGURE 1 THE LIFE CYCLE OF PLASMODIUM FALCIPARUM IN MAN



1 sporozoite enters liver cell; **2** merozoite penetrates red blood cell, **3** ring stage trophozoite; **4** schizont stage; **5** merozoites released; **5a** merozoites re invade red blood cells; **6** macrogametocytes; **7** microgametocytes; **6a** macrogamete; **7a** gametocytes taken into stomach with blood meal; **7b** microgametes; **8** zygote; **9** ookinete penetrates midgut wall of mosquito to develop into an oocyst, **10** sporogony; **11** oocyst ruptures to release sporozoites which enter salivary gland; **12** sporozoites in salivary gland are injected into man with saliva of mosquito.

host's blood stream where they remain for 15 to 60 minutes [Sinden & Smith 1982]. Parasites move in the bloodstream to the liver where they are either taken up by Kupffer cells and then actively invade hepatocytes [Smith et al. 1981] or they enter hepatocytes directly [Shortt 1948].

Once in the hepatocytes the parasite grows and develops into an exoerythrocytic schizont [Garnham 1966]. As the exoerythrocytic schizont grows, nuclear division occurs and there is an increase in cytoplasmic volume, producing in the case of P. falciparum around 30,000 merozoites from each sporozoite. When mature the schizont and host cell rupture releasing merozoites into the blood stream [Garnham 1966]. The pre-erythrocytic cycle in human malaria takes between 6 and 15 days depending on the species. The time between entry of sporozoites into hepatocytes and the release of merozoites is characteristic of each species or strain of parasite. In P. falciparum and P. malariae infections, tissue schizogony follows sporozoite invasion. In P. vivax and probably P. ovale infections a proportion of the sporozoites are thought to transform into dormant stages (hypnozoites) which are responsible for producing relapses [Krotoski et al. 1982 A & B]. The stimulus for the resumption of growth of hypnozoites is not known but Bray & Garnham (1982) suggest that this is somehow predetermined, and the time varies in different strains of P. vivax and P. ovale.

When merozoites are released from the tissue into the circulation they rapidly enter erythrocytes and begin an asexual cycle of parasite multiplication. The merozoites contain an

apical complex which, after orientation comes into contact with a site on the erythrocyte surface which is probably a specific receptor for that species of malaria. These receptors may be associated with the Duffy blood group antigens for P. vivax [Miller et al. 1975] and glycophorin for P. falciparum [Miller et al. 1977, Perkins 1981].

The merozoite attaches to the erythrocyte membrane which after thickening forms a junction with the plasma membrane of the merozoite [Aikawa et al. 1978]. The merozoite releases material from the rhoptries and micronemes, causing an invagination of the erythrocyte membrane, after which the junction moves round the parasite which enters the invagination until it lies completely enclosed in the parasitophorous vacuole [Dvorak et al. 1975, Aikawa et al. 1978]. During the process of invagination the surface coat of the merozoite is lost and appears to remain outside the erythrocyte [Bannister et al. 1975, Miller et al. 1975].

After entry into the erythrocyte the merozoite develops a vacuole and becomes a ring form which ingests small amounts of host cytoplasm (largely haemoglobin) and grows into an amoeboid trophozoite which rounds up as it matures. Asexual multiplication begins with the division of the parasite nucleus. The parasite then segments to form a schizont containing 8 to 32 merozoites (depending on species) which regain their surface coat [Bannister et al. 1977]. The asexual erythrocytic cycle is relatively synchronous in the natural host: one cycle takes 48 hours in P. falciparum, P. vivax and P. ovale and 72 hours in P. malariae. In synchronous

infections the rupture of infected cells and merozoite release is associated with the characteristic fever and acute symptoms of malaria.

The trigger for gametocytogenesis is unclear but some merozoites enter erythrocytes and develop into sexual stages (female macrogometocytes and male microgametocytes). If the mature gametocytes are taken up into the midgut of a female mosquito they are released from the red cells and undergo gametogenesis. This results in the fertilization of a macrogamete by a microgamete to form a zygote (which is thought to be diploid; the parasite is haploid during the rest of the life cycle) which transforms into a motile ookinete which crosses the mid gut epithelium and develops into an oocyst between the gut epithelial layer and the basal lamina of the mosquito midgut wall [Sinden & Strong 1978]. The sporozoites then move to and enter the lumen of the salivary gland. When they have matured into infective forms, the sporozoites penetrate further into the salivary glands [Vanderberg 1975] and are able to infect the next suitable host that the mosquito bites when flowing in the saliva into the wound and the cycle is repeated.

The Clinical Symptoms

P. vivax and *P. ovale* rarely cause anything other than temporary morbidity, while infection with *P. malariae* frequently results in nephrosis, splenomegaly is also common and the spleen may reach considerable size. *P. falciparum*, however, often causes death of the host. In synchronous infections the rupture of infected cells and release of merozoites is associated with a

fever and acute symptoms. If the parasitaemia is allowed to rise unchallenged by natural or acquired resistance or by drug treatment the patient will become severely ill. After an incubation period of between 9 and 14 days (for P. falciparum) the patient develops headaches, general aches and pains, nausea and gastro-intestinal disturbances. As the infection progresses these symptoms become more intense and the patient frequently becomes anxious and confused. At this stage the fever is irregular with no distinct periodicity. As the nausea and gastro-intestinal disturbances become more severe, the spleen becomes enlarged and tender, the liver enlarges and pulmonary involvement occurs. Anaemia develops due to the destruction of both parasitised and non parasitised erythrocytes [Rosenberg et al. 1973, Seed and Kreier 1980]. The release of intracellular debris during the rupture of infected cells may form immune complexes which affect renal function and can lead to complete renal failure. If treated these symptoms quickly subside but if not, severe complications may develop.

When P. falciparum infects an erythrocyte it induces changes in the red cell surface causing it to become adherent to the endothelial cells of the deep tissue vasculature, resulting in the sequestration of erythrocytes containing mature parasites which protects these stages from passage through the spleen [Luse and Miller 1971], and capillaries can become completely blocked by parasitised erythrocytes. Cerebral dysfunction is the most common severe symptom of P. falciparum infection in man. Cerebral malaria may begin slowly after the initial symptoms or develop suddenly resulting in convulsions, numerous nervous disorders and

eventually coma. The typical histopathological feature of cerebral malaria is the blockage of cerebral venules and capillaries with erythrocytes containing late trophozoites and schizonts. Only a small number of patients who die of cerebral malaria have high peripheral parasitaemias or very severe anaemia. There is some dispute over the actual aetiology of cerebral malaria [Reviewed by Warrell 1987].

Drug Treatment

There are several groups of antimalarial compounds which are used for the treatment of malarial infections, to prevent the establishment of infection or to prevent transmission of the parasite.

These compounds include pyrimethamine, quinine, mefloquine, proguanil and chlor proguanil, the 4 aminoquinolines e.g. chloroquine and amodiaquine, the 8 aminoquinolines e.g. primaquine, and the sulphones and sulphonamides. Antimalarial drugs have a selective action on different phases of the parasite life cycle. Causal prophylactic drugs prevent the establishment of the parasite in the liver. Schizontocidal drugs act on the parasite in the red blood cell, and tissue schizontocides act on the exoerythrocytic stage of the parasite. Drugs with gametocytocidal activity destroy the sexual forms of the parasite and sporontocidal drugs inhibit the development of the oocysts in the mosquito such that no sporozoites are produced and the mosquito cannot transmit the infection.

Quinine has fast acting schizontocidal properties and has been one of the most effective drugs in the treatment of malaria

since it was introduced in the early 17th century. During the 1930's and 1940's quinine was replaced by synthetic antimalarials such as mepacrine and chloroquine. With the increasing resistance of some malarial parasites to a number of synthetic antimalarials quinine has again become more widely used. Resistance to quinine has been reported in various parts of the tropics but it has not become a widespread problem.

Drug resistance in malaria is defined 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" [W.H.O. 1965].

Chloroquine is a rapid blood schizontocide and also destroys gametocytes of P. vivax, P. malariae and P. ovale. It can be used both as a preventative and as a curative drug. Chloroquine resistance in P. falciparum has become widespread in Asia, is established in South America and East Africa and has been reported in Central Africa. In most of Africa the parasite shows a decrease in susceptibility to chloroquine.

At present only P. falciparum has developed resistance to chloroquine and it remains the drug of choice in the treatment of other malarial infections as it is relatively inexpensive, well tolerated, safe and fast acting.

Proguanil can be used both for the treatment and prevention of malarial infections. It has a slow schizontocidal action on the erythrocytic forms of malaria parasites and is also active against the primary tissue phase of P. falciparum and has

sporontocidal activity against this species. Some resistance to proguanil has been reported in Malaysia, New Guinea and East Africa. Chlorproguanil is a closely related drug with similar properties.

Pyrimethamine has similar properties to proguanil. It is a slow blood schizontocide and shows considerable activity on primary tissue forms of P. falciparum; it also has some activity against P. vivax, and has sporontocidal properties. Resistance of P. falciparum to pyrimethamine has been reported in parts of E. Africa, W. Africa and the far East, but only in limited areas. Resistance in P. vivax appeared rapidly and is now widespread. Cross resistance between pyrimethamine and proguanil is also apparent.

Primaquine is one of the most important drugs in use that can give a radical cure of P. vivax and P. ovale as it is a tissue schizontocide destroying liver forms and so preventing relapses. It is active against gametocytes but not against other blood stage parasites. The use of primaquine is limited due to several severe side effects.

Sulphones and sulphonamides were occasionally used for the treatment of malaria in the 1940's but they had a number of drawbacks. During the 1960's long acting sulphonamides were introduced and these were found to be useful in treating infections of chloroquine resistant P. falciparum. Sulphadoxine has been particularly useful when used in combination with pyrimethamine, but resistance to this combination has been reported.

Mefloquine is one of the more recent antimalarials and as yet no serious side effects have been noted. Primary drug resistance however, has been reported in Thailand and also cases of reduced sensitivity in Thailand and the Philippines. Strains of P. falciparum resistant to mefloquine are also known to exist in areas even before the drug was used there. Therefore it has been recommended that mefloqine is only used in combination with other antimalarials to delay the development of mefloquine resistance.

A number of drugs with antimalarial properties are currently under development. Q inghaosu, which is isolated from the Chinese medicinal herb, Artemisia annua, has been found to be effective against P. vivax and P. falciparum. Of particular interest is that Quinghaosu has been found to be equally effective against chloroquine sensitive and resistant strains of P. falciparum. Other drugs under development include Halofantrine [Smith, Kline & French], Triozine [Beechams], Clindamycin [Upjohn] and Napthoquinone[Wellcome].

Acquired Immunity to Malaria

The general features of immunity to malaria have been extensively reviewed [Talioferro 1929, Brown 1969, McGregor 1972, Playfair 1982, Deans and Cohen 1983]. It appears that in man resistance to malaria does occur, but is slow to develop. In endemic areas the most serious clinical disease and the greatest prevalence of malaria related deaths are seen in children aged between 6 months and 5 years [McGregor et al. 1956]. With increasing age the disease becomes less severe despite continued

exposure to the parasite. In adults the level of blood parasites is generally very low and the clinical effects of the disease only rarely seen. It appears that a variety of immune responses are elicited during a malaria infection, a large variety of antibodies are produced and there is considerable stimulation of non-specific reticulo-endothelial functions. The immune response may be directed against the sporozoite, the exoerythrocytic stages, the asexual erythrocytic stage and gametocytes. Immunity is strongly stage specific and declines rapidly if the host does not receive frequent antigenic challenge.

The Cellular Response

There is substantial evidence to suggest that T cells are involved in acquired immunity to malaria. Infection with *P. berghei* has been shown to become much more severe in athymic rats, [Brown *et. al.* 1968, Spira *et al.* 1970] and a normally mild strain of *P. yoelii* becomes lethal in athymic nude mice [Clark and Allison 1974, Weinbaum *et al.* 1976]. Evidence for the involvement of T cells in immunity to malaria is also provided by the observation that in animals resistance can be adoptively transferred with immune spleen cells [Phillips 1970] and with purified T cells [Jaywardena *et al.* 1982].

In human malaria patients the numbers of T cells in the peripheral circulation are reduced both relatively and absolutely [Wyler 1976, Wells *et. al.* 1979, Troy — Blomberg *et. al.* 1983A]. An alteration in the ratios between different T cell subsets has also been reported [Troy-Blomberg *et al.* 1983B, 1984]. T cells

show a proliferative response *in vitro* after stimulation with *P. falciparum* antigens, [Troy-Blomberg *et al.* 1983B, 1984], but the response is weak and short lived. Patients with clinical malaria for more than 8 days often show reduced antimalarial proliferative responses while other responses such as to T cell mitogens are the same as for patients with a malarial infection of less than 8 days, indicating suppression of the specific antimalarial T cell response [Brasseur *et al.* 1983, Troy-Blomberg *et al.* 1984). This immunosupression may account for the apparent increased susceptibility of children with malaria to other infections such as measles and gastroenteritis [Hommel 1981].

A number of roles have been suggested for T cells in the immune response to malaria. Brown and Phillips (1971) have indicated a helper function in the production of antimalarial antibody and Weinbaum *et al.* 1976 have shown that a large amount of IgG production in malaria infected mice is T cell dependent.

It is thought that T cells play a role in the recruitment and activation of macrophages as T cell deficient mice showed no increase in phagocytosis and very little splenomegaly during malaria infections while B cell deficient mice behaved normally [Roberts & Weidanz 1979]. This is also suggested by the findings of Coleman *et al.* (1976) who showed a decrease in the numbers of peritoneal macrophages and also detected a macrophage migration inhibition factor (MIF) in spleen cells of mice with acute malaria infections.

Activated macrophages may also mediate parasite death by the release of factors which can kill intracellular parasites [Clark

et al. 1981, Allison & Eugui 1982]. These include tumour necrosis factor (TNF) and lymphocyte activating factor (LAF). Ockenhouse and Shear (1984) have also shown that macrophages activated in vivo and in vitro can kill P. yoelii by the extracellular release of hydrogen peroxide in a phagocytosis associated respiratory burst.

An increase in natural killer cell activity has been reported in children with acute P. falciparum infections [Greenwood et al. 1977]. Ojo Amaize et al. (1981) also found that natural killer cell activity and y-interferon (Y IFN) levels in malaria infected children correlated with increasing parasitaemias. Brown et al. (1986) however, state that there is no evidence to suggest that natural killer cells contribute to parasite killing. Y IFN is produced in vitro when lymphocytes from malaria patients are stimulated with P. falciparum antigen [Troy-Blomberg 1985 in Brown 1986]. Ockenhouse et. al. (1984) have shown that when macrophages are activated by Y IFN they can kill intraerythrocytic parasites in the absence of antibody.

Changes also occur in monocyte-macrophage cells during a malaria infection. It was assumed for many years that the major defence against malaria was phagocytosis. Talioferro (1929) showed that during the parasitic crisis there is an increase in phagocytosis of parasitized erythrocytes and malarial debris. In rodent malaria the phagocytic activity of splenic macrophages can be 20 to 50 times greater than that of normal macrophages [Zuckerman, 1977]. In the spleen the accumulation of macrophages has been shown to be due to a soluble chemotactic factor, thought to originate from lymphocytes [Wyler & Gallin 1977]. Increased

activity and numbers of macrophages have been found in the bone marrow [Frankenburg et al. 1980) and also in livers of mice with malaria, this was most marked in non-lethal infections [Dock rell et al. 1980].

Non Specific Acquired Immunity

There is evidence that the course of a malarial infection can be affected by the action of unrelated agents on the immune system. These include Propiobacterium acnea [Nussenzweig 1967; Clark et al 1977], BCG [Clark et al. 1976], endotoxin [Gregor 1969, Martin et al. 1967], Concanavalin A, lipopolysaccharide, diethystilboestrol [Cottrell et al. 1977], Coxiella burnetti extract [Clark 1979] and freeze thawed Toxoplasma gondii tachyzoites (Omata et al. 1981]. These agents may act through the activation of macrophages, either to increase phagocytosis [Nussenzweig 1967] or to cause production of new cells of the monocyte-macrophage series. These new cells are sensitive to an endotoxin like substance of malaria [Clark 1978, Gauci et. al. 1982] causing the production of tumour necrosis factor [Clark et. al 1981; Taverne et al. 1981, Clark & Hunt 1983, Dockrell & Playfair 1983] or superoxide ions [Allison & Eugui 1982] which damage intraerythrocytic parasites.

Intracellular death of asexual parasites is observed during the crisis period of a malarial infection, especially in laboratory malaria models. A non-antibody, non dialysable factor causing intraerythrocytic death of P. falciparum in vitro has been identified in some sera from immune Sudanese adults [Jensen et

al. 1982A, 1983A]. They found a stronger association between clinical immunity and in vitro serum inhibition with this crisis form factor than with IgG. However some Ig from individuals could induce crisis forms in vitro.

The Humoral Response

The circumsporozoite precipitin reaction (CSP) has been used to demonstrate the presence of antisporozoite antibodies [Vanderberg et al. 1969]. The correlation between the CSP reaction and immunity is thought to be poor [Spitaln y and Nussenzweig 1973] but Yoshida et al. (1980) using monoclonal antibodies to the surface of P. berghei sporozoites have shown that the antigens involved in protective immunity and CSP reaction are identical. These monoclonal antibodies have also been shown to give complete protection against sporozoite challenge in mice [Potocnjak et al. 1980]. Similar findings have also been obtained with P. knowlesi [Cochrane et al. 1982] P. vivax and P. falciparum [Nardin et al. 1982].

The role of specific antibodies in immunity to malaria has also been indicated by B cell transfer experiments in which B cell enriched populations of immune spleen cells have been used to transfer immunity in P. chabaudi [McDonald and Phillips 1978] and P. berghei [Ferraroni and Speer 1982, Gravely and Kreier 1976]. Jayawardena et al. (1979), Weinbaum et al. (1976) and Roberts et al. (1977) have also shown that mice lacking B cells have more severe malaria infections than normal mice.

Immunity has also been passively transferred using whole immune serum in a variety of animal models [for example, Diggs & Osler (1969, 1975), Phillips (1970), Brown and Phillips (1974), Hamburger & Kreier (1975) Playfair and De Souza (1979)]. The protective activity of immune serum has been shown to be due to the immunoglobulin fraction in animals [Diggs & Osler 1969, Phillips & Jones 1972, Green & Kreier 1978, Reese and Motyl 1979] and humans [Cohen et al. 1961, Cohen & McGregor 1963].

Considerable variation is, however, seen in passive transfer experiments, especially in rodent malaria and seems to be related to the dose of immune serum given and to the timing of the serum collection. Total protection against homologous challenge has been reported in recipients of hyperimmune serum [Jayawardena et al 1975 , Golenser et al. 1975] but it is more usual to observe the protective activity of immune serum as a delay in the onset of a patent parasitaemia of recipients [Diggs and Osler 1969, Briggs et al. 1968, Brown & Phillips 1974, Jayawardena et al. 1978].

In vitro studies with merozoites have shown that antibody acts mainly by blocking the invasion of red cells by merozoites [Cohen et al. 1969, Cohen & Butcher 1970, 1971). In the case of P. know lesi sera from immune animals caused agglutination of merozoites [Miller et al. 1975, Butcher & Cohen 1972]. The results of this agglutination test have been shown to correlate well with the results of transfer experiment [Butcher et al. 1978] but not always with functional immunity [Miller et al. 1977]. Antimalarial antibodies have also been shown to block or reverse the cytoadherence of parasite infected (RBCS) in vivo [David et al. 1983].

↑ red blood cells

Extracellular gametes, zygotes and ookinetes have been shown to be susceptible to antibody injested during the blood meal. This antibody has no effect on the intracellular gametocyte in the vertebrate host. It can agglutinate gametes in the mosquito gut, blocking fertilization [Gwadz 1976, Carter et al. 1979]. Antibody can also bind to the zygote surface and block the development of zygotes into ookinetes [Carter et al. 1984]. Antibody can block the infection of mosquitoes and transmission of malaria to new vertebrate hosts, but this has no direct beneficial effect for the infected vertebrate host.

Antibody mediated phagocytosis of free parasites and of schizont infected erythrocytes has been demonstrated in vitro [Celada et al. 1982, Khusmith et al. 1982, Kharazmi & Jepsen 1984]. Brown and Smalley (1980) have also demonstrated antibody dependent cellular cytotoxicity (ADCC) against P. falciparum infected erythrocytes. It has also been shown that during a malaria infection large amounts of immunoglobulin which appear to have no specificity for malarial antigens are produced [Targett & Voller 1965, Abele et al. 1965].

The antigens recognised by these non specific immunoglobulins include antigens of heart, thyroid and gastric parietal cells [Shaper et al. 1968], lymphocytes [Wells et al. 1980], and erythrocytes [Rosenberg et al. 1973, Ronai et al. 1981, Zouali et al. 1982, Wahlgren et al. 1983]. High titres of antibodies to rheumatoid factor [Shaper et al. 1968, Greenwood et al. 1971] and nuclear components [Greenwood et al. 1970, Adu et al. 1982], have also been shown to be associated with malaria infections.

The appearance of different kinds of autoantibodies in the serum of malaria patients has been attributed to polyclonal B cell activation by mitogenic factors [Greenwood & Vick 1975, Greenwood et al. 1979] and is T cell dependent.

Development of a Malarial Vaccine

Three different life cycle stages are potential targets for a vaccine against malaria; they are sporozoites, asexual blood stages parasites and sexual forms.

A completely effective sporozoite vaccine would prevent the establishment of the parasite in the host following a bite by an infected mosquito. Because acquired immunity to malaria is stage specific the recipient of the vaccine would still be susceptible to infection by asexual blood stage parasites, for example by the transfusion of malarious blood. If the immunity induced against sporozoites was incomplete some sporozoites would invade hepatocytes and a low level parasitaemia could occur with the production of gametocytes so that the host would still be infective to mosquitoes.

A vaccine aimed at the asexual blood stage parasites would restrict their multiplication. The third type of vaccine could induce serum antibodies in the human host to block fertilization of female gametes by male gametes or inactivate the fertilized zygote or ookinete in the mosquito gut. This type of vaccine could block malaria transmission but offers no protection to the host against sporozoites, hepatic forms or asexual blood stage parasites.

Most vaccine research has concentrated on finding a protective antibody and identifying the antigen which elicits this response. To be a potential vaccine candidate an antigen must be accessible to antibodies, the antigen-antibody interaction should lead to destruction of the parasite and the antigen should either be conserved in the natural parasite population or show only limited antigenic diversity so that several of the alternative antigenic forms could be administered at once. It would also be an advantage if the antigens were stable, for example they should not show variation when exposed to antibody.

Sporozoite Vaccine

Protective immunity to rodent, monkey [Cochrane et al. 1980] and human malaria [Clyde et al. 1975] has been promoted by vaccination with gamma-irradiated sporozoites. Nussenzweig et al. (1969) demonstrated that part of this protective immunity was due to antibody. This antibody reacts with the surface of sporozoites giving rise to the circumsporozoite precipitin (CSP) reaction, [Vanderberg et al. 1969].

Yoshida et al. (1980) raised monoclonal antibodies to the surface of P. berghei sporozoites and showed that the antigens involved in protective immunity and the CSP reaction were identical. Similar findings have been obtained with P. knowlesi [Cochrane et al. 1982], P. vivax and P. falciparum [Nardin et al. 1982]. The CSP protein is thought to be involved in the invasion of sporozoites into host cells because, in the presence of fab. fragments, sporozoites did not attach to target cells [Hollingdale et al. 1982, 1984]. Ellis et al. (1983) cloned

gene fragments encoding for the P. knowelsi CSP protein and Ozaki et al. (1983) described the sequence of the entire gene which was found to encode a protein with a repeating amino acid sequence (12 amino acids repeated 12 times) containing the epitope which bound protective monoclonal antibodies. The gene encoding the P. falciparum CS protein has also been sequenced [Dame et al. 1984, Enea et al. 1984] and found to contain 41 tandem repeats of 2 tetrapeptides. When the entire CSP gene sequences of P. falciparum and P. knowlesi were compared two regions of homology were found, one on either side of the repeats. Synthetic peptides from the repeat region of the P. falciparum CSP protein were immunogenic in mice and rabbits. The antibody produced blocked the invasion of human hepatoma cells by sporozoites in vitro [Ballou et al. 1985, Zavala et al. 1985].

The P. know lesi CSP gene has been inserted into the vaccinia virus genome and cells infected with this recombinant virus have been shown to synthesize polypeptides that react with a monoclonal antibody against the repeat epitope [Smith et al. 1984].

Similar studies have been carried out with the P. falciparum CSP gene using Escherichia coli [Young et al. 1985]. The expressed peptides were used to immunize mice and high titres of antibodies which reacted with the sporozoite surface and blocked sporozoite invasion of hepatoma cells in vitro were produced. Similar results were obtained when part of the P. vivax CSP protein was inserted into yeast expression vectors [Barr et al. 1987].

Herrington et al. (1987) report the development of a vaccine which was found to be safe in man and to stimulate protective

antibodies. A 12 amino acid synthetic peptide $(NANP)_3$ comprising the immunodominant epitope of the P. falciparum circumsporozoite protein was conjugated to tetanus toxoid and used to immunize human volunteers. Aluminium hydroxide was used as an adjuvant. Three vaccinees and 4 unimmunized controls were challenged with P. falciparum sporozoites. Blood stage parasites were detected in all controls by day 10. Two vaccinees who did become infected did not develop a parasitaemia until day 11 while the third vaccinee did not show parasites or symptoms during the 29 day observation period.

Ballou et al. (1987) have reported the results of a trial in humans of a recombinant DNA P. falciparum vaccine containing multiple NANP repeats, with this vaccine protection also appeared to correlate with antibody levels.

Asexual blood stage vaccine

Asexual blood stage parasites are suitable candidates for a malaria vaccine as they are the main targets of specific acquired immunity [Reviewed by Cohen 1982]. This is also the parasite stage which causes the most pathology.

Monkeys have been successfully immunized against human malaria using parasites cultured in vitro [Reese et al. 1978]. The need to use human serum and erythrocytes for the in vitro cultivation of asexual bloodstage parasites make this method unsuitable for use with humans. This is due to the large amounts of material which would be required, problems associated with

erythrocyte antigens, and the possibility that the human sera and erythrocytes may be contaminated with infectious agents. This method also requires the use of an adjuvant which also makes it unacceptable for use in humans.

Plasmodium falciparum infection stimulates a number of immune responses to a large number of antigens, and therefore there is a need to identify those antigens which induce protective immunity.

A number of antigens have been identified on the surface of infected RBCS, some of which show considerable diversity [Howard et al. 1986 B]. Other antigens appear to be more conserved; these include an antigen associated with the micronemes within metozoites (the RESA antigen) which has been described by Coppel et al. (1984) and Cowman et al. (1984). This 155 kDa antigen is inserted into the erythrocyte membrane at the time of merozoite invasion [Brown et al. 1985] and antiserum to this protein inhibits parasite growth in vitro.

Collins et al. (1986) report that partial sterilizing immunity was achieved in some Aotus monkeys when they were immunized with a genetically engineered fragment of the RESA antigen. Patarroyo et al. (1987) did not achieve protection when using a corresponding peptide sequence (SPf 155.1) to immunize Aotus monkeys. This group, however, do report that a combination of 3 partially protective peptides (SPf 35.1, SPf 55.1 and SPf 83.1) gave complete or almost complete protection. These peptides are fragments from molecules specific for the late schizont and merozoite stages of P. falciparum and have molecular masses of 35 kDa, 55 kDa and 83 kDa.

Marsh and Howard (1986) have evidence for an antigenically conserved epitope expressed on P. falciparum infected erythrocytes in Gambian children. Children susceptible to reinfection with P. falciparum did not appear to make antibodies to this epitope. Serum antibodies from immune adults did, however, bind to this epitope. It may, therefore, be a target for a protective immune response.

Antimalarial antibodies from monkeys can block or reverse the cytoadherence of parasite infected RBCS in vivo [David et al. 1983], and in vitro [Udeinya et. al. 1983]. The parasite antigen responsible for cytoadherence is therefore a suitable target for a malaria vaccine. Immunization with this antigen may induce the production of specific antibodies which would block in vivo cytoadherence and lead to the destruction of parasitised cells by the spleen.

Parasite antigens expressed on the surface of infected erythrocytes are important targets for vaccination as they are exposed to antibody for relatively long periods and so may prove effective targets even with low levels of low avidity antibody.

Merozoite Vaccine

Merozoites are the only asexual blood stage malaria parasite directly accessible to antibodies, and therefore antigens of the merozoite surface are potential targets for immune responses which would prevent merozoite invasion of erythrocytes. A number of in vitro studies have shown that antimalarial sera from infected or immunized monkeys or human patients [Mitchell et al. 1975, Green

et al. 1981] or monoclonal antibodies specific for merozoite antigens [Epstein et al. 1981, Deans et al. 1982, Deans 1984, Holder & Freeman 1984] can block merozoite invasion of erythrocytes. Merozoites are only directly exposed to the immune system for 1-2 minutes [Dvorak et al. 1975] before they enter a new erythrocyte. A vaccine aimed at merozoite surface antigens would depend upon antibodies either agglutinating merozoites or blocking specific receptors to prevent invasion of new erythrocytes in this short time. Holder et al. (1985) have sequenced the surface glycoprotein of P. falciparum merozoites and it will now be possible to immunize monkeys with defined peptides corresponding to antigenically conserved portions of this merozoite protein.

Sexual stage vaccine

A vaccine against sexual forms has the potential to block malaria transmission by preventing fertilization of gametes [Gwadz 1976, Carter et al. 1979] or preventing zygote or ookinete development in the mosquito [Carter et al. 1984] but has no direct beneficial effect on the host.

Monoclonal antibodies have been raised to specific surface antigens on gametes and zygotes [Rener et al. 1980, Kaushal et al. 1983, Rener et al. 1983). Some monoclonal antibodies block fertilization while others prevent zygote development. The surface antigens which can act as targets for this immunity have been identified in P. gallinaceum [Rener et al. 1980, Kaushal et al. 1983] and in P. falciparum [Rener et al. 1983].

There are a number of important points and problems which will determine the usefulness of a malaria vaccine. These include the initial vaccine regime required. This will depend on the immune status of an individual and may vary with age and circumstances. For example a visitor to a malarious area would require a vaccine which induced complete immunity to infection. It may however, be more beneficial to use, for example, a vaccine against asexual stage parasites to reduce morbidity in susceptible individuals such as children and pregnant women living in malarious areas. The vaccine would protect against initial contacts with the parasite and repeated infections would boost the immune response so that it would remain at protective levels. Other factors to be considered include, the duration of the protective effect, the need for boosters, the role of natural infections as boosters of induced immunity and the effect of antigenic variation. Thought must also be given to the cost and stability of a vaccine.

It is likely that many epidemiological situations will probably require a vaccine which will be a combination of an antisporozoite vaccine, with one or several asexual stage antigens and also a transmission blocking component.

In Vitro Cultivation of Malaria Parasites

Cultivation of the Asexual Erythrocytic Stages of P. falciparum

There have been many attempts to cultivate malaria parasites in vitro. The first published work is that of Bass & Johns (1912) who describe the development of P. falciparum from the ring to the

schizont stage in whole blood supplemented with dextrose or maltose. However, it was not until 1976 that the continuous *in vitro* cultivation of the asexual erythrocytic stage of *P. falciparum* was achieved by two separate groups; Trager & Jensen (1976), and Haynes *et al.* (1976). The method of Trager & Jensen (1976) has become widely used and subsequent studies by Trager & Jensen (1977, 1978) have shown that the following conditions are necessary for the successful continuous cultivation of *P. falciparum*. A thin settled layer of human erythrocytes, covered with a layer (2-4 mm deep) of RPMI 1640 medium supplemented with 25 mM Hepes [N-2-hydroxyethlpiperazine-N'-2-ethanesulfuric acid] and 10% human serum. The correct ratio of RBCS to medium is obtained using 10% RBC suspension. Trager and Jensen suggest two methods, both providing the above conditions.

In the flow vial method a continuous slow flow of medium is maintained over the cells. The advantage of this method is that daily attention is not required. In this method an atmosphere of 7% CO_2, 5% O_2 and 88% N_2 is kept flowing constantly over the culture.

In the second method the cultures are maintained in plastic petri dishes and the medium is changed manually once a day. An atmosphere of approximately 3% CO_2, 17% O_2 and 80% N_2 is provided by a candle jar. It is interesting to note that Scheibel *et al.* (1979) have shown that the best parasite growth was promoted by atmospheric conditions of 3% CO_2, 2% O_2 and 95% N_2.

In both of the methods of Trager & Jensen described above subculturing is carried out by mixing an appropriate quantity of

infected cells from a culture with a quantity of freshly washed uninfected cells. Trager and Jensen (1978) report that erythrocytes from A, AB or O blood groups can be used successfully for the cultivation of malaria parasites (blood group B erythrocytes were not tested). Some variation has however, been found among donors in the ability of their erythrocytes to support parasite growth. Erythrocytes stored at $4^{o}C$ in ACD (acid citrate dextrose) or CPD (citrate phosphate dextrose) preservatives for 21 days (no longer suitable for transfusion and therefore considered outdated by blood banks) have been found to be as suitable as freshly collected erythrocytes in supporting parasite growth [Capps and Jensen 1983].

The culture medium used by Haynes et al. (1976) was supplemented medium 199, while Trager and Jensen (1976) used RPMI 1640 which was originally designed for the in vitro cultivation of human leucocytes [Moore et al. 1967]. Divo & Jensen (1982A) compared the suitability of media for the cultivation of P. falciparum and found that RPMI 1640 was superior to Hams F12 and medium 199 supplemented with either Eagles or Hanks salts and therefore RPMI 1640 has remained the medium of choice. To maintain the pH of cultures between 7.3 and 7.5 (to obtain optimal parasite growth) Trager & Jensen (1976) used RPMI supplemented with 25 mM Hepes and 0.2% sodium bicarbonate ($NaHCO_3$). Malaria parasites produce lactic acid (Jensen et al. 1983B) which causes a pH change in cultures; Hepes is a zwitterion buffer system which augments the CO_2 bicarbonate buffer system of the medium and also has the advantage of maintaining a stable pH when cultures are removed from the CO_2 enriched atmosphere required for parasite growth.

Trager & Jensen (1976) first achieved continuous cultivation of malaria parasites with RPMI containing 10 or 15% AB serum, but any serum compatible with the erythrocytes being used is suitable. Jensen (1979) found that samples of human serum vary in their ability to support parasite growth some samples being effective in supporting growth at a concentration of 5% or less.

Divo and Jensen (1982B) have shown that if human sera from 20 or more donors are pooled, 5% serum will support optimal parasite growth. The requirement for human sera does however present certain problems. In endemic areas human sera may be inhibitory to cultures due to the presence of antimalarial antibodies, antimalarial drugs or other inhibitory factors [Jensen et al. 1982B]. Even in developed countries human sera is expensive and can be difficult to obtain. Furthermore there is always the risk that sera may be contaminated with an infectious agent. A suitable replacement for human serum is therefore desirable.

Initial attempts to grow P. falciparum using commercially available animal sera were not successful [Jensen et al. 1979], but Ifediba & Vanderberg (1980) have reported that calf serum supplemented with neopeptone or proteose peptone No. 3 will support parasite growth as well as 10% human serum. However parasites do appear to require an extensive adaptation period before continuous growth can be maintained.

Divo & Jensen (1982B) tested freshly collected bovine, porcine, equine and ovine sera along with three types of processed human serum and found that only bovine serum supplemented with neopeptone could support continuous parasite growth and this was at reduced levels.

Butcher (1979) reports the growth of a line of P. falciparum in RPMI supplemented with 10% horse serum, after an initial adaptation period; and Sax & Reichman (1980) successfully grew parasites in medium containing 5% pooled rabbit serum.

Willet and Canfield (1984) successfully cultivated 2 isolates of P. falciparum for 4 weeks in serum free medium, though growth rates were lower than in control cultures with human serum.

Recently Grun and Weidanz (1987) have successfully used commercially obtained pooled human and animal (horse, calf and bovine) serum to cultivate P. falciparum after the removal of antibodies reactive with human erythrocytes.

The Cultivation of the Asexual Erythrocytic Stages of Other Species of Plasmodium.

The erythrocytic stages of 4 species of malaria parasites of rhesus monkeys have now been successfully cultivated using modifications of the method of Trager & Jensen (1976). Namely P. fragile [Chin et al. 1979], P. cynomolgi [Nguyen-Dinh et al. 1981], P. inui [Nguyen-Dinh et al 1980] and P. know lsi [Wickham 1980]. Culture systems allowing continuous cultivation of the erythrocytic stages of the other human malaria parasites, P. vivax, P. malariae and P. ovale with the same ease as P. falciparum have not yet been established. Ray Chowderey et. al. (1979) report the growth of P. malariae in mixed cultures with P. falciparum; but the establishment of pure cultures of P. malariae or the use of this mixed culture system in further studies has not been reported.

Larrong et al. (1981) reported the cultivation of P. vivax for 43 days using the method of Trager & Jensen (1976) but with higher levels of glucose in the RPMI and more frequent medium change. Brockelman et al. (1985) were unable to repeat this success and suggested that the poor re-invasion rates observed were due to the predeliction of P. vivax for immature erythrocytes [Kitchen 1939]. The findings of Mons et al. (1987) also suggest that this requirement for reticulocytes is the main limiting factor in the re-invasion of P. vivax. Reticulocytes comprise only 1-2% of the total erythrocyte population. Therefore considering this and the above work, the report by Renapurkar et al. (1983) of cultures of P. vivax achieving parasitaemias of 10% is confusing. P. berghei also preferentially infects reticulocytes and this parasite has been successfully maintained in continuous culture [Janse et al. 1984] by the use of a suspension culture method and by diluting cultures with rat blood containing a high proportion of reticulocytes.

Cultivation of sporogonic stages

Early studies on the in vitro transformation of ookinetes to oocysts and then to sporozoites were somewhat limited due to the problems of obtaining sporogonic stages from mosquitoes. However, this transformation has been obtained in successive short term cultures [Reviewed by Vanderberg et al. 1975].

Alger (1968) and Ro sales-Ronquillo and Silverman (1974) have described the transformation of gametocytes to ookinetes in vitro but these methods were either not reproducible or gave low numbers of ookinetes. The method used by Weiss & Vanderberg (1977) for

the transformation of P. berghei gametocytes to ookinetes has been analysed and improved by Janse et al. (1985) and Sinden et al. (1985) and is now routinely used. A similar method has been developed for the cultivation of P. gallinaceum. [Aikawa et al. 1984]. Carter et. al. (1987) report a method for the production of mature ookinetes of P. falciparum developed from the method used for P. berghei.

Cultivation of Exoerythrocytic Stages

The exoerythrocytic stages of avian malaria parasites have been successfully cultured in vitro for over 20 years (Reviewed Beaudoin 1977), but it is only recently that the exoerythrocytic stages of mammalian malaria parasites have been successfully cultured in vitro.

Strome et al. (1979) reported that sporozoites of P. berghei successfully entered rat embryonic liver and brain cells and underwent some development. These findings have been confirmed by Sinden & Smith (1980) who also found that fibroblasts from embryonic rats were susceptible to invasion by P. berghei sporozoites.

It was Hollingdale et al. (1981) who first demonstrated the complete in vitro development of the exoerythrocytic stages of P. berghei. They found that a human embryonic lung cell line (W138) was susceptible to invasion by P. berghei sporozoites and would support the complete cycle of development with the release of merozoites [Hollingdale et al. 1983].

It has subsequently been shown that P. yoelii will develop in primary hepatocyte cultures of Thamnomys gazellae [Mazier et al. 1982] and that P. berghei also develops in a hepatoma cell line (HEP G2-A16) [Hollingdale et al. 1983].

Mazier et al. (1984) have succeeded in obtaining the complete exoerythrocytic development of P. vivax, in human hepatocytes, with the production of infective merozoites. Smith et al. (1984) have also successfully cultivated the exoerythrocytic stages of P. falciparum in human hepatocytes.

It is interesting to note that Hollingdale et al. (1985) have described the cultivation of 2 populations of exoerythrocytic parasites of P. vivax in the cell line Hep G2-A16. One population was composed of dividing schizonts which released merozoites, the second population did not divide and remained after the primary schizonts had disappeared from the culture. Hollingdale et al. (1985) suggest that this second non-dividing population may be hypnozoites.

Gametocyte Production of P. falciparum in vitro

Row (1928) reported the presence of gametocytes in cultures of P. falciparum maintained according to the method of Bass & Johns (1912). A number of workers have subsequently reported the appearance of gametocytes in other P. falciparum culture systems, [Haynes et al. 1976; Smalley 1976, Mitchell et. al. 1976, Trager, 1971, Phillips et al. 1976, Trager & Jensen, 1976, 1978, Jensen 1979]. Since the introduction of the technique of Trager & Jensen (1976), there have been several studies attempting to elucidate the factors controlling gametocytogenesis and

gametocyte maturation. Infective gametocytes have been obtained from cultures by the addition of cyclic adenosine monophosphate (cAMP) (Kaushal et al. 1980) or hypoxanthine [Ifediba & Vanderberg 1981] and by maintaining cultures at a constant temperature of 37-38°C [Ponnudurai et al. 1982] during the 10 days or so necessary for the complete development of P. falciparum gametocytes in vitro. The optimal conditions permitting the production of gametocytes from all isolates in continuous culture still have not been fully explained but it appears that conditions which are unfavourable to asexual forms favour gametocyte production, [Carter & Miller 1979 , Phillips et al. 1978]. The situation regarding the precise conditions necessary for the production and maturation of gametocytes remains unclear but it would appear that environmental factors control the induction of gametogenesis to some extent.

Different isolates of P. falciparum show intrinsic differences in their capacity for gametocyte production [Brockelman 1982, Chin & Collins 1980]. It has also been shown that there is a tendency for individual isolates of P. falciparum to lose the ability to produce gametocytes in vitro; the time taken for this appears to vary from a few weeks to over a year [Ponnudurai et al. 1982] and is characteristic of a particular isolate.

Graves et al. (1984a) have shown that clones derived from a single isolate were also widely different in their capacity for gametocyte production. The failure of certain isolates to produce gametocytes in vitro may be due to the loss or suppression of part of the genome responsible for gametocyte production or as a result of unfavourable culture conditions.

Diversity in Plasmodium falciparum

The slow rate at which natural immunity to malaria develops gave the first indications that considerable diversity exists between malaria parasites, not only between species but also among organisms belonging to a single species.

It is now well documented that natural populations of malaria parasites are phenotypically diverse with respect to a variety of characters such as drug resistance, patterns of proteins on 2-dimensional electrophoresis, levels of gametocytogenesis, isozyme type and importantly antigenic phenotype [Reviewed by Walliker 1983].

Carter and McGregor (1973) showed that Gambian isolates of P. falciparum often exhibited more than one electrophoretic form of one or more enzymes. Studies involving isolates from other parts of the world have shown that they also contain mixtures of enzyme types [Sanderson et al. 1981, Thaithong et al. 1981, Hempelmann et al. 1981].

The extent of the diversity found within single isolates of P. falciparum has been further investigated by cloning studies. Rosario (1981) prepared clones from a single isolate of P. falciparum from Thailand and showed that, while the original uncloned material exhibited a mixture of several enzyme variants, single clones showed only a single form of each enzyme. This has been confirmed by the findings of Thaithong et al. (1984) and Graves et al. (1984A).

Isolates from different parts of the world have been found to exhibit differences in susceptibility to antimalarials. Thaithong et al. (1981) demonstrated that isolates of P. falciparum from Thailand were less sensitive to chloroquine and pyrimethamine than isolates from West Africa. Cloning studies have demonstrated the occurrence of parasites of differing drug sensitivities within an isolate. Graves et al. (1984A) found that clones prepared from a single Brazilian isolate differed up to 1000 fold in their resistance to pyrimethamine and Thaithong (1983, 1984), Trager et al. (1981) and Kyle-Webster et al. (1985) all prepared clones which exhibited variation in susceptibility to antimalarials.

Tait (1981) has shown, using 2-dimensional electrophoresis, that protein variations exist between isolates from different countries and also between isolates from one region of Thailand. Fenton et al. (1985) examined proteins in 2 isolates of P. falciparum and found that while certain proteins possessed more than one form in uncloned material, only single forms were present in clones. This has been confirmed by the findings of Thaithong et al. (1984).

Considerable antigenic diversity has also been shown to exist between isolates of P. falciparum [McBride et al. 1982, Wilson et al. 1969, 1975, Wilson 1980, Hommel et al. 1983, Thaithong et al. 1984, Graves et al. 1985, McBride et al. 1985, Howard et al. 1986 A&B, Marsh & Howard 1986].

Wilson et al. (1969, 1975) and Wilson (1980) demonstrated the diversity of S. antigens between different isolates. S. antigens are soluble malarial proteins which are released into the plasma or culture medium when parasitised erythrocytes rupture to

release merozoites. These antigens also remain stable and immunoreactive after treatment at 100°C [Wilson et al. 1969].

Wilson et al. (1969) examined sera from malaria patients in the Gambia and found that individual patients had up to five S. antigens in their serum at any one time; the diversity of S. antigens within isolates has also been demonstrated by Winchell et al. (1984) Anders et al. (1983) and Howard et al. (1986A).

McBride et al. (1982) raised monoclonal antibodies to two isolates of P. falciparum from Thailand. These monoclonal antibodies were then used in an immunofluorescence test against P. falciparum isolates from various countries. Some antibodies gave positive results with all isolates tested showing that they recognised antigens common to all isolates; other antibodies gave positive results with some isolates but not with others. Within individual isolates some parasites gave positive results with a given antibody while others were negative, indicating antigenic diversity not only between isolates but also within individual isolates.

Marsh & Howard (1986) used an antibody mediated agglutination assay to show antigenic diversity in isolates of P. falciparum in the Gambia. Serum samples taken from a group of children in the convalescent stage of malaria reacted with PRBCS from the same child but not usually with infected cells from other children. Adult serum from the same area often reacted with PRBCS from all the children indicating that the childrens erythrocytes also showed shared determinants. The adult serum was also shown to contain antibodies to several isolates.

Clones prepared from a single isolate of P. falciparum have also been shown to differ in the presence or absence of knobs [Trager et al. 1981, Thaithong et al. 1984, Green et al. 1985] and gametocyte producing ability [Trager and Jensen 1981, Graves et al. 1984B].

The development of the technique of Trager & Jensen (1976) for the in vitro cultivation of the asexual erythrocytic stages of P. falciparum has been a major breakthrough in the field of malaria research, but problems associated with the adaptation of freshly isolated parasites to culture still remain. Most isolates grow poorly during the first weeks of cultivation, [Trager & Jensen 1976, Chin & Collins 1980] and only some can be established sucessfully in culture.

Trager (1982) has suggested that difficulties of adaption may be due to technical inadequacy rather than because of any inherent properties of the parasite. However, a decline in parasite numbers is frequently observed during the first 1-6 weeks of cultivation suggesting that parasite selection is taking place. In view of the considerable diversity found within individual isolates of P. falciparum it would seem likely that when an isolate is slow to adapt to culture, selection is occurring, in so far as only a proportion of the original parasite population may be able to make the transition to continuous growth in culture. This view is supported by the findings of a number of workers. Isolates cultured in vitro have been found to show changes in drug sensitivity (Jensen et al. 1981) and the ability to produce gametocytes [Ponnudurai et al. 1982). Howard et al (1986A) were able to

identify more 3H glycine labelled S. antigens in isolates analysed directly from patients than were found by Anders et al. (1983) and Winchell et al. (1984) in culture-adapted P. falciparum isolates.

Rosario (1981) found that an isolate from Thailand contained 2 forms of glucose phosphate isomerase, GPI 1 and GPI 2 and 2 forms of adenosine deaminase ADA1 and ADA2. After 2 weeks in vitro cultivation ADA 2 could not be detected and after 5 weeks of cultivation GPI 2 could not be detected either. The cultivation of isolates bearing knobs (K+) on the infected erythrocyte membrane has also been shown to lead to the loss of these knob structures (K^-) [Langreth et al. 1979]. This loss of knobs was the first change observed in P. falciparum as a result of long term in vitro cultivation and has been shown to correlate with the loss of the property of cytoadherence to human endothelial cells [Udeinya et al. 1983] or amelonatic melanoma cells [Schmidt et al. 1982]. K^- variants have also been found to be less virulent than K+ parasites in intact Aotus monkeys [Lanners & Trager 1984; Green et. al. 1985] but K^- parasites grow more rapidly than K+ parasites in vitro [Motyl & Reese 1983]. This is important as it implies that the parasites studied and cultured in vitro are those most able to survive under culture conditions rather than those best adapted for life in the host. It is therefore probable that most lines of P. falciparum which have been adapted to culture do not represent all the genetically distinct clones present in the original isolate.

The conservation of the entire parasite population is an important aim if P. falciparum cultures are to be used for

biochemical analysis, for drug screening or for antigen production.

The use of Feeder Cells

It has been reported that some cell types and hybridomas grow more vigorously and show extended life in vitro when cultivated in association with other cells (feeder cells).

Shaffer et al. (1953) reported that a liver suspension prepared from chick embryos supported better multiplication of Entamoeba histolytica than cell suspensions prepared from other embryonic chick organs.

Puck and Marcus (1955) described the effects of irradiated monolayers of HeLa cells on the cloning of these cells in vitro.

Michalopoulos et al. (1979) described the use of a feeder cell layer of human fibroblasts to cultivate parenchymal hepatocytes from adult rats, and Rheinwold and Green (1975) reported the growth of human diploid epidermal cells on a feeder cell layer of fibroblasts (T3 cells). Ishii et al. (1981) also used human fibroblasts as feeder cells for mouse lymphoma (L1210) cells. L1210 cells have also been successfully cultivated using a feeder cell layer of mouse macrophages [Nathan & Terry 1975], 3T3 cells and L cells [Tanapat et al. 1978].

The use of feeder cells in the cultivation of bloodstream form trypanosomes is now well established. Hirumi et al. (1977) used a feeder cell layer of fibroblast-like cells isolated from bovine blood or tissue to cultivate hematozoic trypanosomes. Epithelial-type cell lines have also been used successfully as

have some commercially available cell lines from other mammals Hirumi et al. (1980). Brun et al. (1981) have also successfully cultivated bloodstream forms of T. brucei, T. rhodesiense and T. gambiense using feeder cells derived from the embryos of New Zealand white rabbits or the mountain vole Microtus montanus.

The use of feeder cells in the cultivation of malaria parasites has also been reported.

Sulzer and Latore (1977) used feeder cell layers of human embryo lung fibroblasts, human amnion cells, and kidney cells from Rhesus and African green monkeys when culturing P. falciparum, P. vivax and P. malariae to the schizont stage. They reported that no failures in the culture method occurred when feeder cells were used but occasional failures did occur when feeder cells were not used.

Brown and Smalley (1980) reported that a significant increase in the growth of a culture adapted isolate of P. falciparum was observed in cultures containing peripheral mononuclear cells (PBMNS).

Mazier et al. (1984A) have shown that culturing P. falciparum in the presence of a feeder cell layer of rodent hepatocytes co-cultivated with a liver epithelial cell line leads to an increase in parasite multiplication. These workers also report that new isolates of P. falciparum appear to adapt to culture more readily in the presence of a feeder cell layer.

Phillips et al. (1987) report that fresh and cryopreserved isolates of P. falciparum can be adapted to continuous in vitro cultivation by the use of feeder cell layers of PBMNS or mouse peritoneal wash cells (PWCS).

The method of Mazier et al. (1984) using rodent hepatocytes as feeder cells is technically complex and not suitable for routine application. This is also true of the method of Phillips et al. (1987) involving the use of PBMNS.

The use of PWCS as feeder cells, as described by these workers is however simple and suitable for routine use.

Experimental Rationale

It is now well documented that natural infections of P. falciparum are mixtures of genetically diverse parasites and it would seem likely that when an isolate is slow to adapt to culture, selection is taking place in so far as only a proportion of the original parasite population is able to make the transition to in vitro cultivation. This is important because it implies that parasites cultured and studied in vitro may not be the most important component of the original infection. It is therefore desirable to adapt isolates as rapidly as possible to in vitro culture and to maintain the parasite population as closely as possible to the original.

It has been shown that isolates of P. falciparum exhibit increased multiplication rates and adapt to culture more readily when cultured in the presence of feeder cells [Mazier et al. 1984, Phillips et al. 1987].

The role played by feeder cells in promoting an increase in parasite multiplication rate and in the adaptation of new isolates of P. falciparum to culture was investigated.

The use of feeder cells was also applied to the development of a technique allowing parasites to be cloned (by limiting dilution) straight from an infected blood sample without first having to adapt the isolate to culture.

CHAPTER 2

MATERIALS AND METHODS

New Isolates of P. falciparum

Blood was collected from infected patients into preservative free heparin (Evans) in PBS pH 7.2 (see Appendix) (10 i.u. ml^{-1} blood). The parasitised blood was stored at $4^{o}C$ until it could be processed.

Normal Human Erythrocytes

Normal human, blood group O rhesus positive red blood cells (NRBCS) were provided by the West of Scotland Blood Transfusion Service. The cells were received as outdated (4 weeks after donation) 450 ml packs of whole blood collected into Citrate Phosphate Dextrose Adenine (CPDA). Aliquots were removed aseptically and centrifuged at 250g for 15 mins and the plasma and buffy coat removed. The NRBS were washed twice in incomplete RPMI 1640 medium (see Appendix) by centrifugation (250g for 15 minutes) and resuspension, before being stored in incomplete medium at $4^{o}C$ for up to 4 weeks prior to use in cultures.

Normal Human AB Serum

Normal human AB serum was provided by the West of Scotland Blood Transfusion Service. This was received deep frozen and was thawed on arrival. It was then dispensed in 10 ml aliquots, frozen and stored at $-70^{o}C$ until required.

Routine Maintenance of P. falciparum in vitro

Parasites were routinely maintained in vitro according to the method of Trager & Jensen (1976) in complete RPMI 1640 (see Appendix).

Parasites were cultured at a 10% haematocrit in 35mm plastic petri dishes (Gibco) and incubated at 37^{o}C in a candle jar. The medium was changed daily and cultures were diluted with NRBCS when the parasitaemia reached 2-3%.

Parasitaemia

The parasitaemia was determined by counting the percentage of infected RBCS in thin blood smears made from cultures or from blood samples. The blood smears were air dried, fixed in absolute methanol (Analar-BDH) and stained in 10% Giemsa's stain (GURR) in buffer, pH 7.2 (see Appendix). Blood smears were examined under oil immersion using a 100 x objective and a X 10 eyepiece lense on a Leitz S.M. Lux binocular microscope.

Low parasitaemias were enumerated by counting the number of parasites observed in a total of 30 fields. If more than 3-4 parasites were observed per field, the number of parasites observed in a total of 500 erythrocytes was counted. Blood smears were considered to be negative if no parasites were observed in a total of 100 fields.

Initiation of Cultures

A) New Isolates

Infected whole blood was centrifuged at 250g for 15 minutes and the plasma and buffy coat removed. The packed RBCS were washed twice in incomplete medium by centrifugation and resuspension. They were then resuspended in complete medium to give a 10% haematocrit and dispensed in 1.5 ml aliquots in 35mm plastic petri dishes.

B) Recovery from stabilate

Parasites were recovered from stabilate by thawing in a 37°C waterbath. The ampoule contents were transferred to a sterile universal tube and diluted with 0.5 mls of 4.5% saline followed by 4.5 mls of 3.5% saline (see Appendix). This was added dropwise with frequent mixing. The parasitised RBCS (PRBCS) were washed twice in incomplete medium, resuspended in complete medium and dispensed as above.

Cryopreservation of Parasites

A) New Isolates

Parasites from patients were cryopreserved using the method of Phillips and Wilson (1978). Blood was diluted 50:50 with a solution of sorbitol/glycerol (see Appendix). The sorbitol/glycerol was added slowly, dropwise with frequent mixing. Aliquots of up to 1 ml of the diluted blood were snap frozen and stored as stabilates in liquid Nitrogen until required.

B) Established Cultures

Cultures with a high proportion of ring stage parasites were centrifuged at 200g for 5 mins and the culture medium removed. The packed PRBCS were resuspended in an equal volume of human AB serum, which was then diluted with sorbitol/glycerol and frozen as above.

Synchronization of P. falciparum erythrocytic stages in culture

Cultures were synchronized according to the method of Lambros

and Vanderberg (1979). Cultures with a high proportion of ring stage parasites were centrifuged at 200g for 5 minutes. The pellet was resuspended in 2.5 mls of 5% D-Sorbitol (see Appendix) per 0.5 ml of packed PRBCS and incubated at room temperature for 5 mins. The PRBCS were washed twice in incomplete medium and returned to culture. The treatment was repeated 33 hours later.

Concentration of P. falciparum schizont infected RBCS

Schizont infected RBCS were concentrated using a modification of the method of Dluzewski et al. (1984). Parasite cultures diluted (in complete medium) to a 5% haemotocrit were layered onto 75% Percoll (Pharmacia) pre-warmed to 37^{o}C and centrifuged at 350 g for 15 minutes. A band of schizont infected RBCS was formed at the medium/Percoll interface which was collected, washed twice in incomplete medium by centrifugation (200g for 5 minutes) and resuspension.

Cloning of P. falciparum by limiting dilution

A suspension of PRBCS was prepared in complete medium so as to contain 0.25 PRBCS per 100 ul at a 2.5% haemotocrit. 200 µl aliquots were dispersed into a 96 well microtitre plate; 100 µl of medium was replaced daily. On days 5, 12 and 17, 100 µl of medium were removed and replaced with 100 µl of a NRBC suspension at 0.5% haemqtocrit. After 21 days a thin blood smear was made from each well and examined for the presence of parasites.

Collection of Mouse Peritoneal Wash Cells (PWCS)

PWCS were collected by peritoneal lavage of 3-6 month old, male or female NIH mice, with 5 mls of ice cold incomplete medium. The cells were collected aseptically by aspiration into a 5 ml syringe with a 21G needle, centrifuged at room temperature for 5 minutes at 200g and resuspended in ice cold complete medium. Cell numbers were evaluated in a haemocytometer and then diluted with complete medium to give the required concentration. The cells were stored on ice throughout the procedure.

Separation of Adherent and Non Adherent PWC populations

Method 1

PWCS were incubated overnight in complete medium at a concentration of 2×10^6 cells ml^{-1} in plastic petri dishes at 37^oC in a candle jar. The following day the non adherent cells were resuspended, by pipette, in the medium in the petri dish, and removed to another petri dish. The number of non adherent cells was counted using a haemocytometer. The remaining adherent cells were washed by the addition and removal of complete medium to the petri dish with a pipette. The adherent cells were then either used *in situ* or were removed using a rubber policeman, resuspended in complete medium and counted using a haemocytometer. The adherent cells were stored on ice during this procedure.

Method 2

Twenty grams of Sephadex G10 (Pharmacia) was rehydrated in excess PBS, swollen at 90^oC for 1 hour and degassed. Most of the

PBS was then removed and the Sephadex autoclaved for 20 minutes. A 20 ml sterile syringe barrel with the plunger removed was loosley packed with 0.5 ml of sterile glass wool. The Sephadex was added and allowed to settle to a depth of 10 cm (the PBS being allowed to run out of the syringe). The column was washed through with 10 mls of warm complete medium. 1 ml of complete medium containing up to 2×10^8 PWCS was added to the column which was then washed through with 25 mls of warm complete medium and the effluent containing the non adherent PWCS collected.

PWC Conditioned Medium

PWCS were incubated in complete medium at a concentration of 2×10^6 cells ml^{-1} at 37°C in 5% CO_2 for 24, 48, 72, 96 or 120 hours. The culture supernatant was then collected, centrifuged at 200g for 5 mins and frozen at -20°C until required.

PWC Conditioned NRBCS

Adherent PWCS were prepared at a concentration of 1×10^6 cells ml^{-1} in complete medium. NRBCS were added to give a 10% haematocrit and incubated at 37°C in a candle jar for 72 hours.

Cultivation of Human Cell Lines

Three human cell lines, Raji, K562 and 143 were grown in complete medium in T-25 tissue culture flasks (NUNC-Gibco Europe) using 5 mls of medium per flask and incubated at 37°C in 5% CO_2 and 95% air. Raji grows in suspension and 2.5 mls of medium was replaced every 3rd day. 143 and K562 both grow attached to the flask and 2.5 mls

of medium was replaced every 2nd day. When the cells became confluent they were trypsinised.

Trypsinisation for transferring Cell Lines

The medium was removed from flasks containing a confluent cell layer. The cells were washed with PBS (pH 7.2), cold 10% trypsin (Northumbria Biologicals Ltd) in PBS was added for 30 seconds and then discarded. The flask was incubated at 37°C in 5% CO_2 until the cells detached (5-15 minutes). The cells were resuspended in complete medium to give the required concentration of cells/ml (1×10^4 for 143, or 3×10^5 for K562).

Irradiation of Cells

Cells were irradiated with 3000 rads from a cobalt source, at a rate of approximately 550 rad/min. The cells were suspended in complete medium.

Cultivation of Microtus montanus cell line

The cells were cultured in Minimum Essential Medium (MEM, see Appendix) in T-25 tissue culture flasks using 5 mls of medium per flask. They were incubated at 37°C in 5% CO_2 and 5 mls of medium was changed daily. When the cells became confluent they were trypsinised, resuspended in 10 mls of medium and divided between 2 flasks.

Cryopreservation of PWCS

PWCS were cryopreserved using a modification of the method of Wells & Price (1983).

200 µl aliquots of PWC suspension at 2 x 10^6 cells ml^{-1} were dispensed in 96 well plates and incubated overnight at 37°C in 5% CO_2. The complete medium was removed and replaced with 150 µl of freezing medium; either RPMI with 10% Foetal Calf Serum (FCS) and 6% DMSO, or FCS with 10% DMSO. The plates were wrapped in cling film and placed inside jiffy bags and frozen at -70°C.

Plates were thawed as described in the text by the addition of 150 µl of warm incomplete medium followed by incubation for 5 minutes at 37°C in 5% CO_2. The freezing medium was removed and replaced with 200 ul of complete medium and the plate incubated as above.

Indirect Fluorescent Antibody Test [IFAT]

Cultures with a high proportion of late trophozoite and schizont stage parasites were used as a source of antigen. The PRBCS were washed 3 times in excess PBS (pH 7.2) (see appendix) by centrifugation (200 g for 5 minutes) and resuspension.

After the last wash the cells were resuspended to less than the original blood volume.

Teflon coated 12 well multitest slides (Flow) were washed in detergent, rinsed in tapwater followed by distilled water and then ethanol and finally hand dried with Kleenex tissues.

Approximately 100 µl of PRBC suspension were pipetted up and down onto the well several times to ensure the well was completely covered. Slides were dried with a hair drier, packed with silica gel and stored at -20°C.

The IFAT used followed the method of used by McBride et. al (1982). Slides were taken from -20°C, immediately fixed in absolute acetone [May and Baker] and air dried. 40 µl of monoclonal antibody dilution (diluted in PBS with 1% sodium azide and bovine serum albumin (BSA)) was added to each well and then incubated for 30 minutes at room temperature in a humid chamber. The slides were washed 3 times in successive baths of PBS, drained and FITC conjugated anti mouse IgG (Miles) diluted 1:25 in PBS containing Evans Blue (1:0 000 w/v) was applied to the slides and incubated for 30 minutes. The slides were again washed and mounted in 1:1 PBS/Glycerol (Merck). Fluorescene was observed using a Leitz microscope. The overhead ultraviolet source was a Wotan HBO-50 Mercury lamp with 2 X KP490 exciting filters and a TK510 dichoric beam splitting mirror and a K515 supression filter.

The slides were examined using a X50 water objective and X12 eyepieces.

The specificities of the monoclonal antibodies used in this test are shown in Table 1.

Table 1

The Specificities of Monoclonal Antibodies used in the IFAT Test

Monoclonal Antibody	Antigen Recognised	stage-specificity
* 7.3-7	gp 200 000	schizonts,
*13.1-2	"	"
* 9.2-6-2	"	"
* 9.5-1-5-1	"	"
*12.1-5-4	"	"
*13.2-3	"	"
* 9.8-4-4-1	"	" common
*13.4-2-1	p 55 000	"
*12.5-1-2	?	"
* 5.1-1-4	p 23 000	most asexuals
* 9.21-4-2	90 000	ER membrane

* in PBS, pH 7.3, with 1% BSA and sodium azide

CHAPTER 3

Observations on the use of peritoneal wash cells as feeder cells for cultures of *P. falciparum*

Introduction

The use of a feeder cell layer in the cultivation of the asexual erythrocytic stages of P. falciparum has been described by Mazier et al. (1984) and Phillips et al. (1987). Both these groups report that new isolates of P. falciparum show increased multiplication rates and adapt to culture more readily when grown in the presence of feeder cells.

Mazier et al. (1984) used a feeder cell layer of rodent hepatocytes co-cultivated with a liver epithelial cell line, while Phillips et al. (1987) used human peripheral blood mononuclear cells. These methods are, however, either technically complex, or involve repeated venepuncture of human donors and therefore are not suitable for routine application. Phillips et al. (1987) also report preliminary observations that mouse PWCS can be used successfully as a feeder cell layer for P. falciparum cultures.

The following work is an investigation into the possibility of using mouse PWCS as a simple and convenient feeder cell which can be used in the routine cultivation of P. falciparum. A number of preliminary investigations into the mode of action of PWCS on cultures of P. falciparum are also reported.

The growth of new and established isolates of P. falciparum with different concentrations of PWCS

Experiment 3:1

This experiment was carried out in order to try to determine

the optimal concentration of PWCS which would promote maximum parasite multiplication. Two isolates of P. falciparum (AF and API) were grown in wells containing 1×10^6, 5×10^5, 1×10^5 or no PWCS/well in 200 µl of complete medium in 96 a well microtitre plate (NUNC) at a 5% haematocrit. Each treatment was carried out in triplicate.

Isolate API had been in continuous culture for 85 days prior to the experiment and AF had only been in culture for 3 days. The starting parasitaemia for strain API was 1.0% and for AF 2.0%. 150 µl of medium from each well were changed daily.

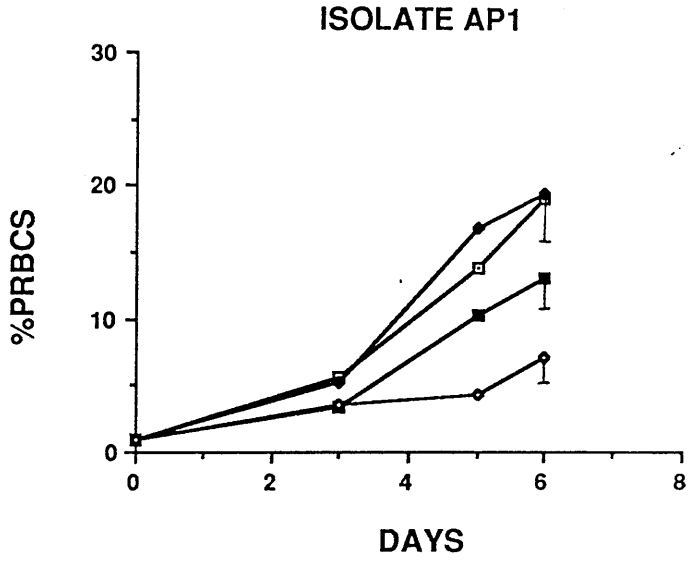
Result

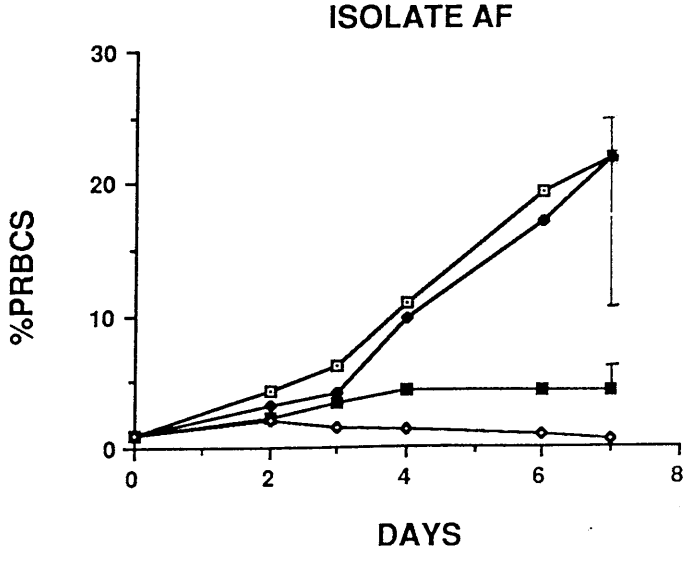
Both isolates showed increased multiplication rates at all PWC concentrations tested when compared with control cultures (no PWCS) (see Figure 3.1). The most parasite multiplication was promoted by 5×10^5 and 1×10^6 PWCS/well. The increase in multiplication rate was most marked with the new isolate AF which reached a mean parasitaemia of 22% on day 7 with 1×10^6 PWCS, compared with a 1% parasitaemia in control cultures. Isolate API reached a mean parasitaemia of 19.5% with 5×10^5 and 1×10^6 PWCS on day 6 compared with 7.1% in control cultures.

Similar results were obtained when the experiment was carried out with new isolates AF and FF and established isolates FMG and K.

FIGURE 3.1 Isolates AF & AP1 were grown in wells containing $1x10^6$ ⊡ $5x10^5$ ◆ , $1x10^5$ ■ or no ◆ PWCS/well in 200ul of complete medium in a 96 well microtitre plate. Each treatment was carried out in triplicate. Bars indicate spread of points

FIGURE 3.1 THE CULTIVATION OF TWO ISOLATES OF P. FALCIPARUM WITH DIFFERENT CONCENTRATIONS OF PWCS.





The relationship between parasitaemia and the number of RBCS/ml in P. falciparum cultures with and without PWCS

Experiment 3.2

This experiment was carried out to confirm that the increase in the number of parasitised RBCS observed when P. falciparum is grown in the presence of PWCS is due to increased numbers of PRBCS and not due to the destruction of non infected RBCS by PWCS, or through spontaneous lysis of RBCS in cultures, thereby giving a false impression of an increasing parasitaemia.

Isolate K was grown in 700 µl of complete medium at a concentration of 2.0×10^8 RBCS ml^{-1} in wells containing 1×10^6 PWCS in a 24 well multiwell plate. Isolate K was also grown under the same conditions without PWCS.

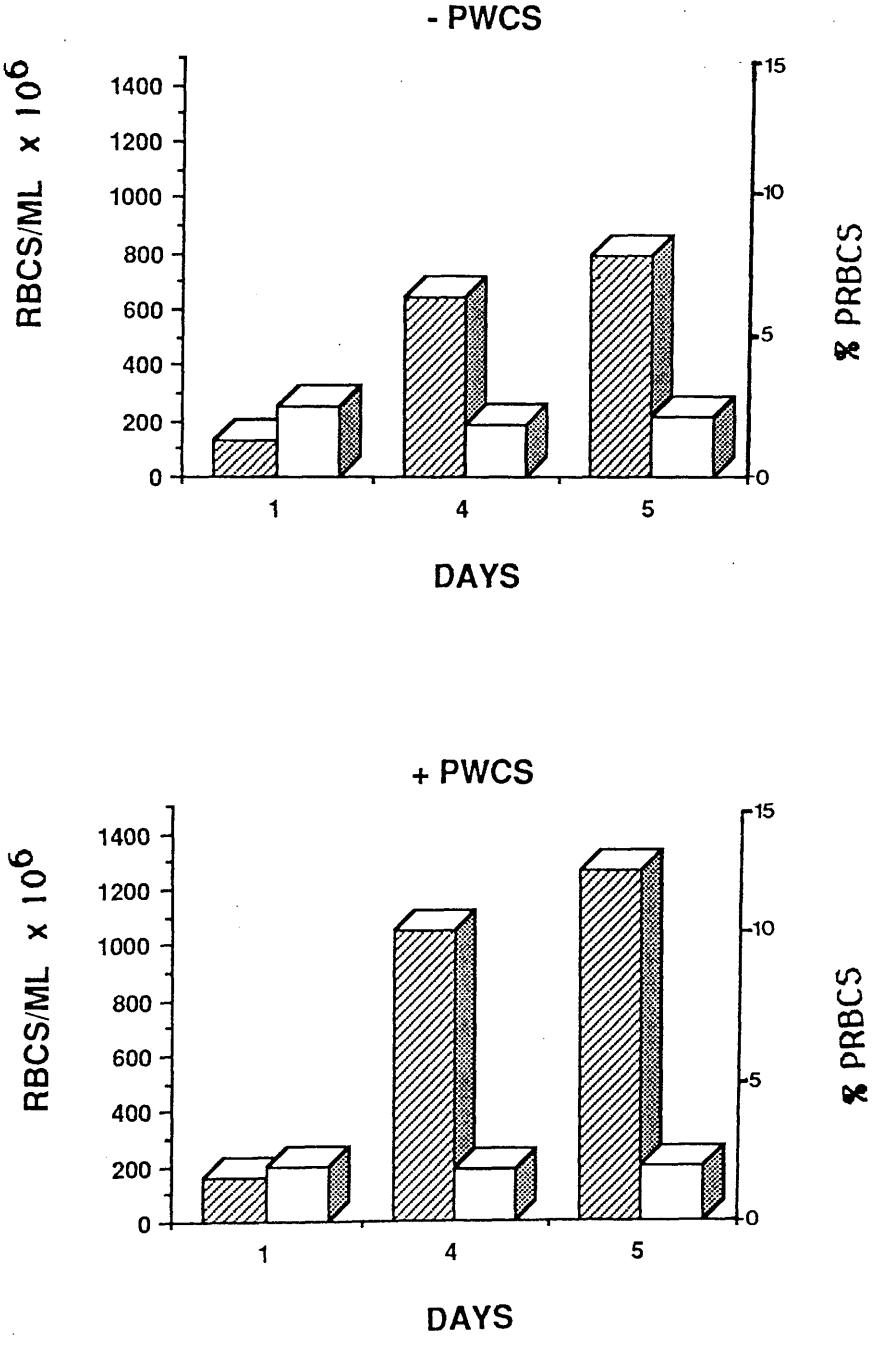
The starting parasitaemia of isolate K was 0.5% and 500 µl of complete medium from each well were replaced daily. Smears were made on days 1, 4 and 5. Two wells from each group (with and without PWCS) were selected on these days and a total RBC count carried out from each. The experiment was repeated using isolate FF with a starting parasitaemia of 1.0%.

Results

The number of RBCS/ml appears to remain relatively constant as the parasitaemia increases, in cultures both with and without PWCS (see Figure 3.2).

FIGURE 3.2 Isolate K was grown at a concentration of $2x10^8$ RBCS/ml in 700µl of complete medium and also in wells containing $1x10^6$ PWCS in a 24 well multiwell plate. Two wells from each group [with and without PWCS] were selected on days 1, 4 and 5 and the total number of % PRBCS ▨ and RBCS/ml □ counted. The data shown is the mean value of the two wells

FIGURE 3.2 THE RELATIONSHIP BETWEEN PARASITAEMIA AND THE NUMBER OF RBCS/ML IN P. FALCIPARUM CULTURES WITH AND WITHOUT PWCS



On day 5 cultures with PWCS had a mean parasitaemia of 12.8% and 1.95 x 10^8 RBC/ml compared with control cultures which had a parasitaemia of 8.0% and 2.1 x 10^8 RBC/ml^{-1}.

A similar result was obtained when the experiment was repeated using isolate FF.

The Growth of P. falciparum with adherent and non-adherent PWCS

Experiment 3.3A

The PWC population is mainly comprised of macrophages and lymphocytes. It is possible to obtain a macrophage enriched cell population by making use of the adherent properties of the macrophages, either by allowing them to adhere to plastic (method 1, see Materials and Methods) or by passing the PWC population through a sephadex G10 column (Method 2, see Materials and Methods). Here the beneficial effect of adherent (macrophage enriched) and non-adherent (macrophage depleted) PWC populations on the growth of 3 isolates (K, FF and AF) of P. falciparum is compared.

Isolate K was considered well adapted to in vitro cultivation, but isolate FF had only been cultured for 4 days prior to the experiment. Isolate AF was used as stabilate prepared directly from an infected blood sample and so had not been cultured prior to this experiment.

Adherent (AD) and non adherent (NAD) PWCS were prepared (using method 1) at a concentration of 5 x 10^5 PWCS/well in 500 µl

aliquots of complete medium in a 24 well multiwell plate. 3 x 500 µl aliquots of complete PWC population were also dispensed at a concentration of 5 x 10^5 PWCS/well, along with 3 x 500 µl aliquots of complete medium. 200 µl aliquots of a PRBC suspension with a parasitaemia of between 0.15% and 2.0% was added to each well giving a final haematocrit of 3.0%. 500 µl of medium from each well were replaced daily.

Result

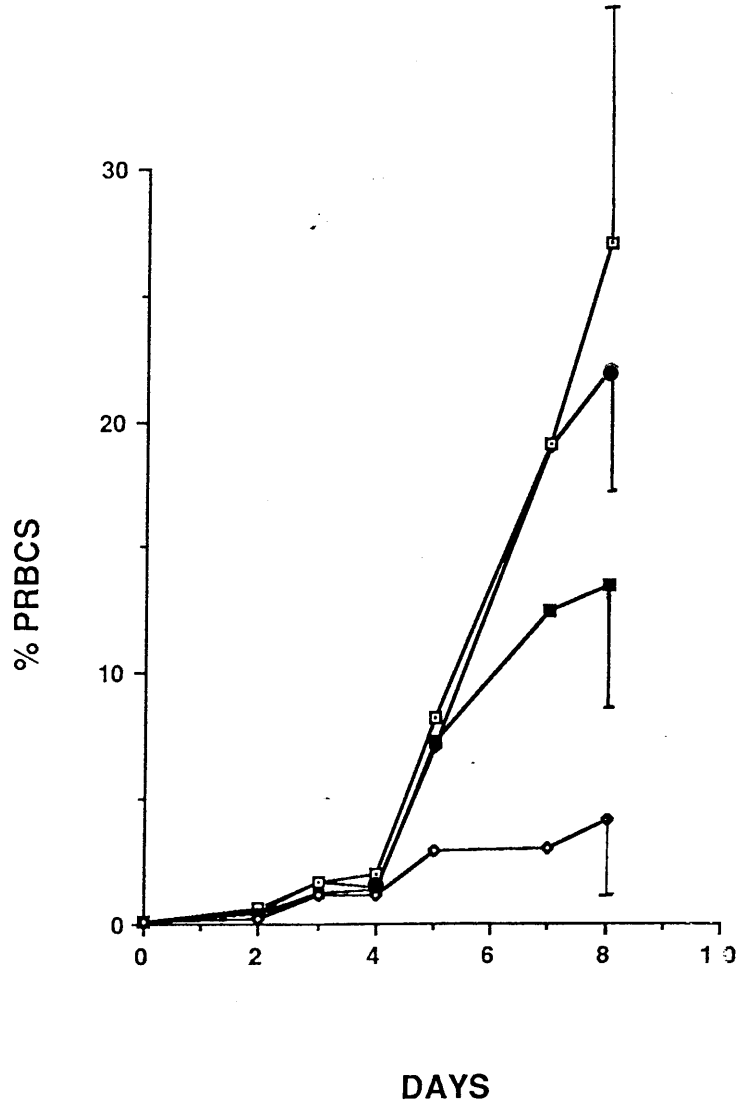
The experiment was carried out a total of 5 times with isolate AF, twice with K and once with FF. Figures 3.3, 3.4 and 3.5 show the results of representative experiments.

Cultures with adherent and complete PWC populations showed an increase in parasite multiplication which was greater than that promoted by the non adherent PWC population. In the case of isolate AF (Figure 3.3) the adherent and complete PWC populations promoted mean parasitaemias of 21.9% and 25.3% respectively by day 8 compared with 8.0% for non-adherent PWCS. The non-adherent PWCS do, however, appear to support more parasite multiplication than control cultures (no cells) which achieved a mean parasitaemia of 5.1% by day 8.

Experiment 3.3B

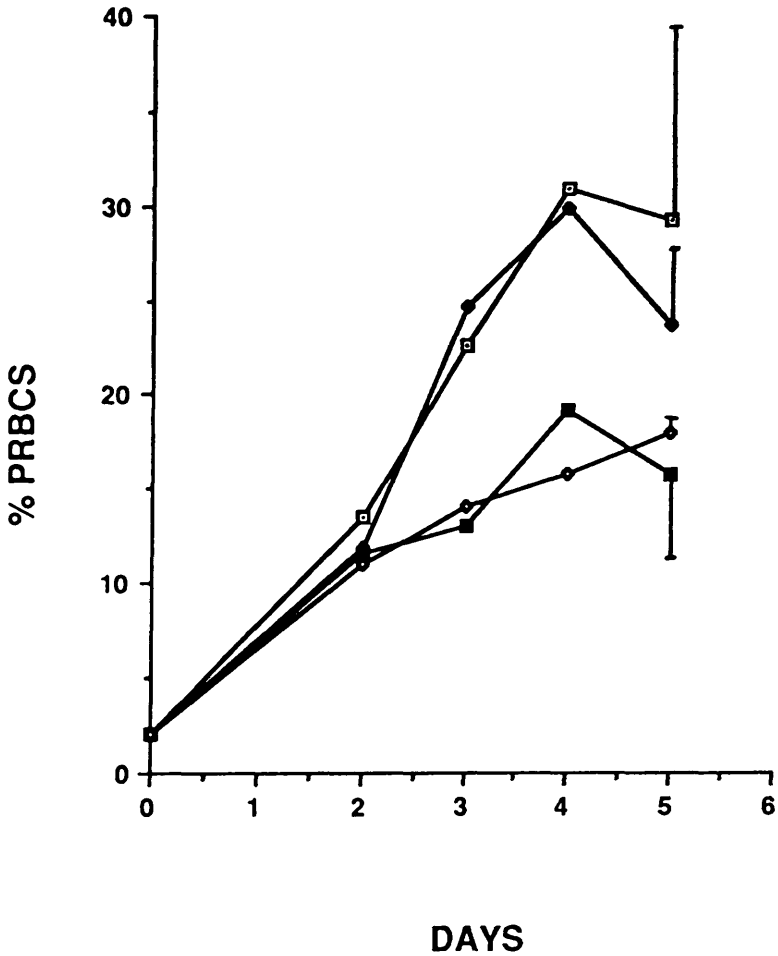
In this experiment the effects of non adherent PWCS prepared by methods 1 and 2 on cultures of *P. falciparum* were compared.

FIGURE 3.3 THE CULTIVATION OF ISOLATE AF WITH ADHERENT AND NON-ADHERENT PWCS.



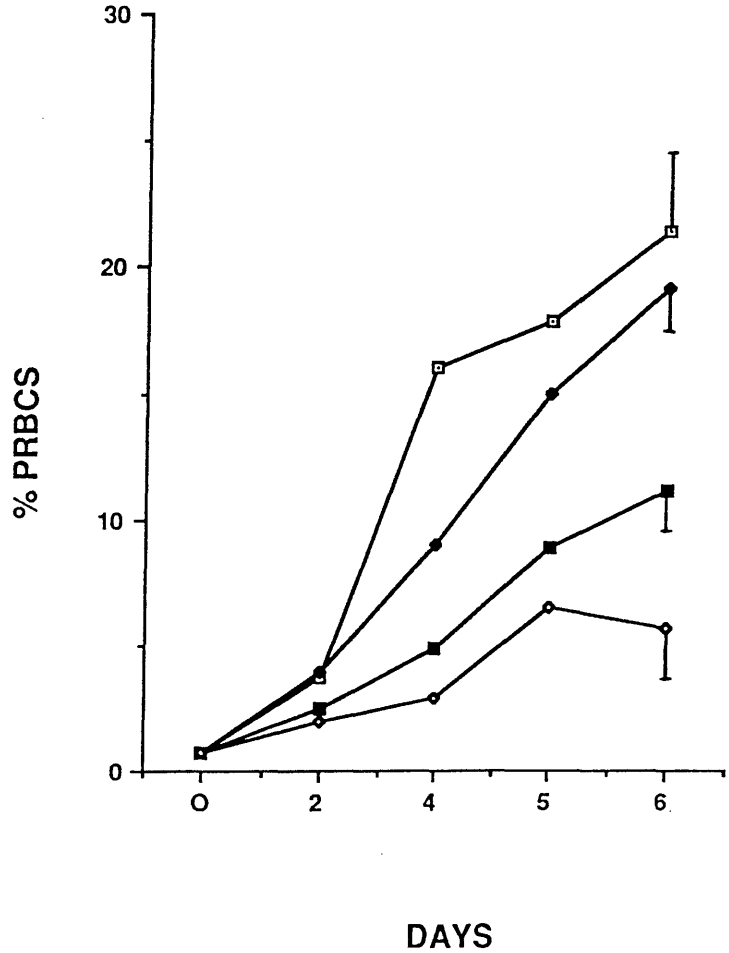
Isolate AF was grown in wells containing $5x10^5$ cells of an adherent ●, non-adherent ■ or complete ⊡ PWC population and also without PWCS ◇ in 500µl aliquots of complete medium in a 24 well multiwell plate. Each treatment was carried out in triplicate. Bars indicate spread of points.

FIGURE 3.4 THE CULTIVATION OF ISOLATE K WITH ADHERENT AND NON-ADHERENT PWCS.



Isolate K was grown in wells containing $5x10^5$ cells of an adherent -●- , non-adherent -■- or complete -◘- PWC population and also without PWCS -◆- in 500µl aliquots of complete medium in a 24 well multiwell plate. Each treatment was carried out in triplicate. Bars indicate spread of points.

FIGURE 3.5 THE CULTIVATION OF ISOLATE FF WITH ADHERENT AND NON-ADHERENT PWCS.



Isolate FF was grown in wells containing $5x10^5$ cells of an adherent -◆- , non-adherent -■- or complete -□- PWC population and also without PWCS -◇- in 500µl aliquots of complete medium in a 24 well multiwell plate. Each treatment was carried out in triplicate. Bars indicate spread of points.

Non-adherent cells were prepared using method 2 and then used at a concentration of 1×10^6 cells ml^{-1} and dispensed in 500 µl aliquots in a 24 well plate. This had been prepared as above, using isolate AF with a starting parasitaemia of 0.4%.

Result

When non-adherent PWCS prepared by methods 1 and 2 were compared they supported mean parasitaemias of 2.9% and 0.9% respectively (see Figure 3.6) on day 4, this declined to 0.37% and 0.3% by day 8. These parasitaemias were lower than those promoted by adherent or complete PWC populations (which achieved mean parasitaemias of 6.3% and 12.4% respectively on day 8) but were slightly higher than control cultures which did not show any parasites by day 8.

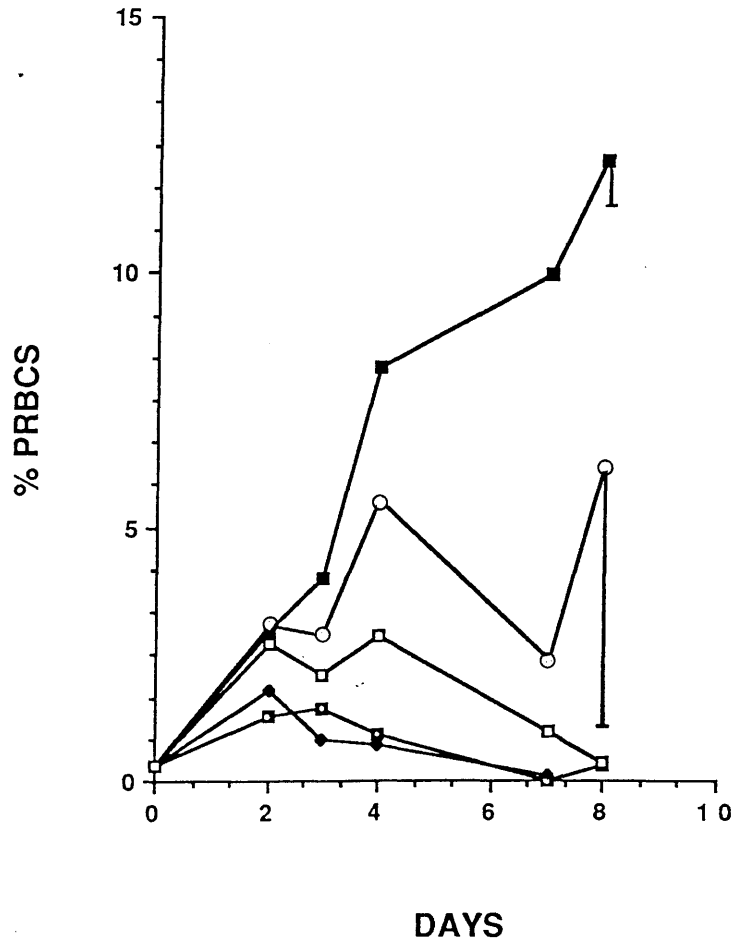
Growth of P. falciparum in PWC conditioned RBCS

Experiment 3.4

In an attempt to determine the mechanism by which PWCS promote an increase in parasite growth, the possibility that they affect the NRBC membrane in such a way as to make it more amenable to merozoite invasion was explored. This was accomplished by culturing parasites with 'normal' and PWC conditioned RBCS.

Three cultures of P. falciparum (isolates BG, RC and FMG) were synchronised according to the method of Lambros and Vanderberg (1979), when the parasites reached the schizont stage

FIGURE 3.6 **THE CULTIVATION OF ISOLATE AF WITH ADHERENT PWCS AND NON-ADHERENT PWCS** [PREPARED USING TWO DIFFERENT METHODS].



Isolate AF was grown in wells containing $5x10^5$ non adherent PWCS which had been prepared prepared using either method 1 -□- or method 2 -□- . Isolate AF was grown with the same number of cells of an adherent -○- or complete -■- PWC population and also without PWCS -◆- , in 500µl aliquots of complete medium in a 24 well multiwell plate. Each treatment was carried out in triplicate. Bars indicate spread of points.

they were collected on a Percoll gradient. The concentrated schizont preparations were diluted with either normal RBCS or with PWC conditioned RBCS and returned to culture at a 10% haematocrit in 1.5 ml aliquots in 35 mm plastic petri dishes. Smears were made from each culture 12 hours later when re-invasion had occurred. 1.5 mls of medium from each culture were replaced daily and smears were made on days 4 and 6.

Result

A summary of results is shown in Table 3.A. The cultures with PWC conditioned RBCS did not appear to be any more effective than cultures with normal RBCS in supporting parasite multiplication (see Figure 3.7).

The effect of separating PWCS from parasitised RBCS on the multiplication rate of P. falciparum

Experiment 3.5A

In order to determine whether PWCS need to be in direct contact with PRBCS to promote an increase in parasite multiplication PWCS were separated from parasitised RBCS using Millicell-HA (Millipore) culture plate inserts which are permeable to macromolecules but not to cells.

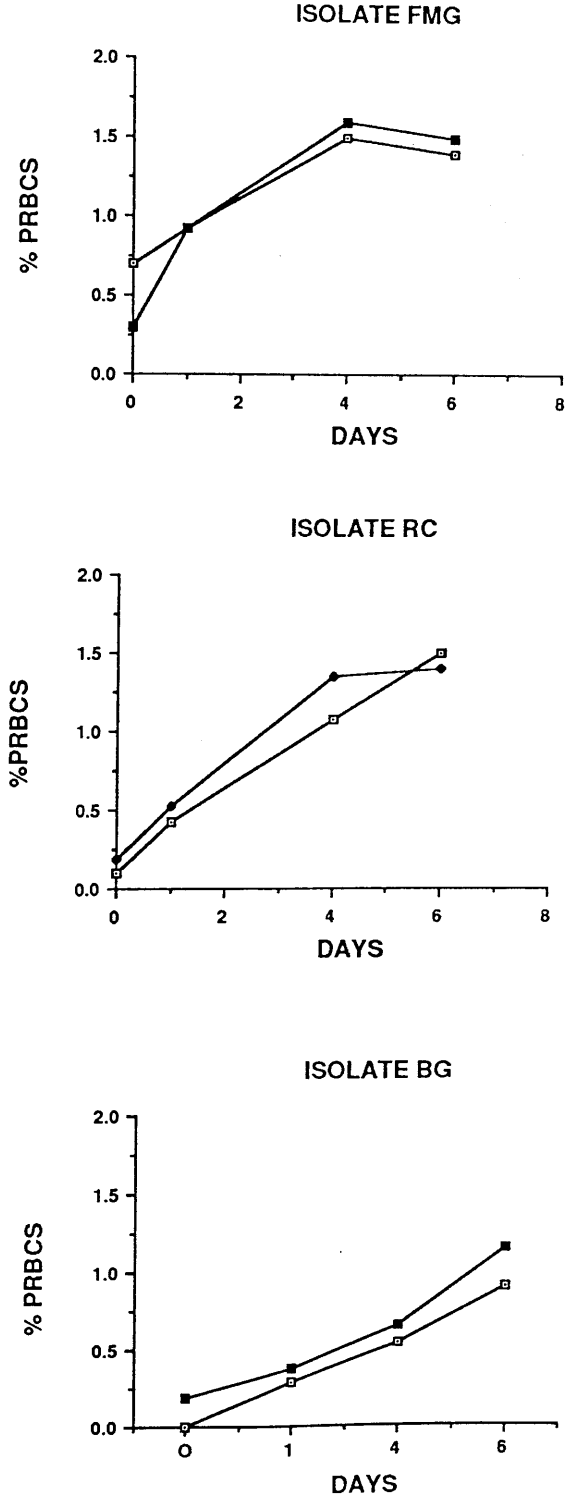
Isolate K was grown at a 10% haematocrit in 500 µl aliquots of complete medium in a 24 well multiwell plate, both with and without 1×10^6 PWC/well. The PWCS were either in direct contact

Table 3A

The growth of P. falciparum in normal and PWC conditioned RBCS

Isolate	% Schizonts after concentration on Percoll	Sample diluted with PWC conditioned RBCS				Sample diluted with normal RBCS			
		Starting parasitaema	12 hrs	4 days	6 days	Starting parasitaema	12 hrs	4 days	6 days
BG	46.6	0.057	0.29	0.54	0.9	0.19	0.37	0.65	1.15
RC	40.9	0.19	0.52	1.35	1.4	0.1	0.43	1.07	1.5
FMG	21.0	0.7	0.93	1.5	1.4	0.3	0.93	1.6	1.5

FIGURE 3.7 THE CULTIVATION OF THREE ISOLATES OF P.FALCIPARUM IN NORMAL AND PWC CONDITIONED RBCS.



Isolates FMG, RC and BG were grown in normal ■ or PWC conditioned ⊡ RBCS in 1.5 ml aliquots of complete medium at a 10% haematocrit in 35mm plastic petri dishes.

with, or were separated from the PRBCS. The PWCS and PRBCS were separated by placing the PWCS in 500µl of complete medium into Millicell-HA culture plate inserts which were then placed inside wells containing parasite cultures.

Each treatment was carried out in triplicate and the starting parasitaemia of K was 0.8%, 450 µl of medium from each well and culture plate insert were replaced daily.

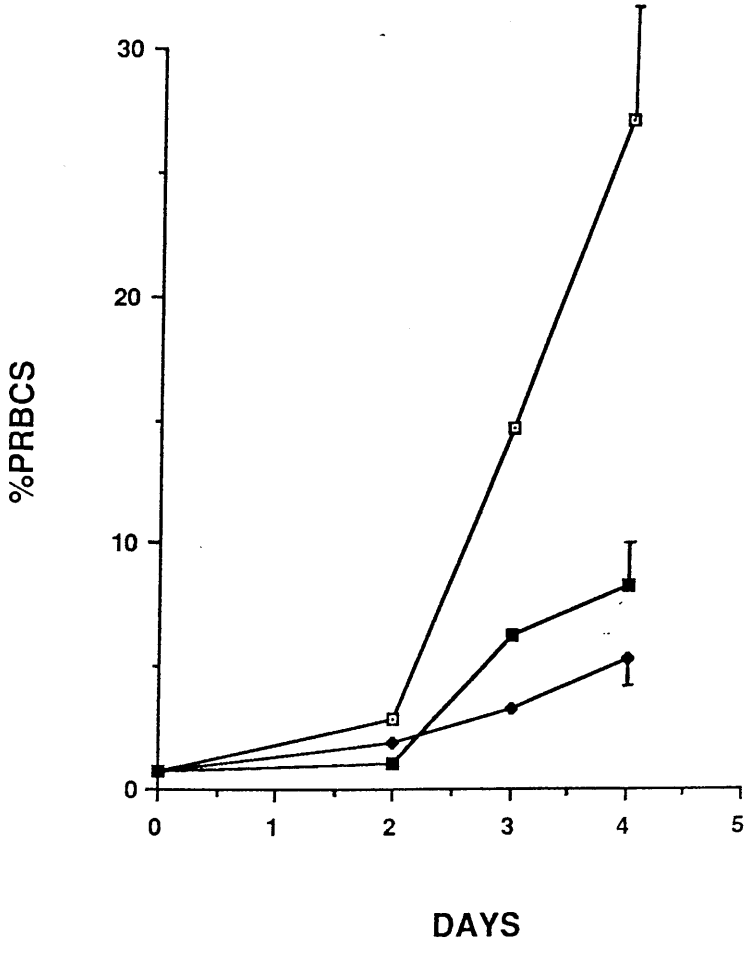
Result

When PWCS were separated from parasite cultures, these cultures reached a mean parasitaemia of 5.2% on day 4. This was lower than control cultures (no inserts or PWCS) at 8.3% or cultures in direct contact with PWC which reached a mean parasitaemia of 27.3% (see Figure 3.8).

Experiment 3.5B

This experiment was carried out to investigate the possibility that the Millicell-HA culture plate inserts were themselves affecting parasite growth. A 24 well plate was prepared as described above but culture plate inserts containing 500 µl of complete medium with no PWCS were also included. Isolate FMG was used with a starting parasitaemia of 1.0%. 450 µl of medium from each well and culture plate insert were replaced daily.

FIGURE 3.8 THE CULTIVATION OF ISOLATE K WITH PWCS AND MILLICELL H-A CULTURE PLATE INSERTS.



Isolate K was grown at a 10% haematocrit in 500µl aliquots of complete medium in a 24 well multiwell plate with $1x10^6$ PWCS/well. The PWCS were either in direct contact with ⊡ or were separated from the PRBCS ◆ by Millicell culture plate inserts . Isolate K was also grown without PWCS ■ . Each treatment was carried out in triplicate. Bars indicate spread of points.

Result

Cultures in which inserts had been placed showed less parasite multiplication than control cultures (no inserts or PWCS) or cultures containing PWCS alone, (see Figure 3.9). On day 3 cultures containing inserts both with and without PWCS reached mean parasitaemias of 1.8% compared with 11.1% in control cultures and 14.8% in cultures which were in direct contact with PWCS.

Experiment 3.5C

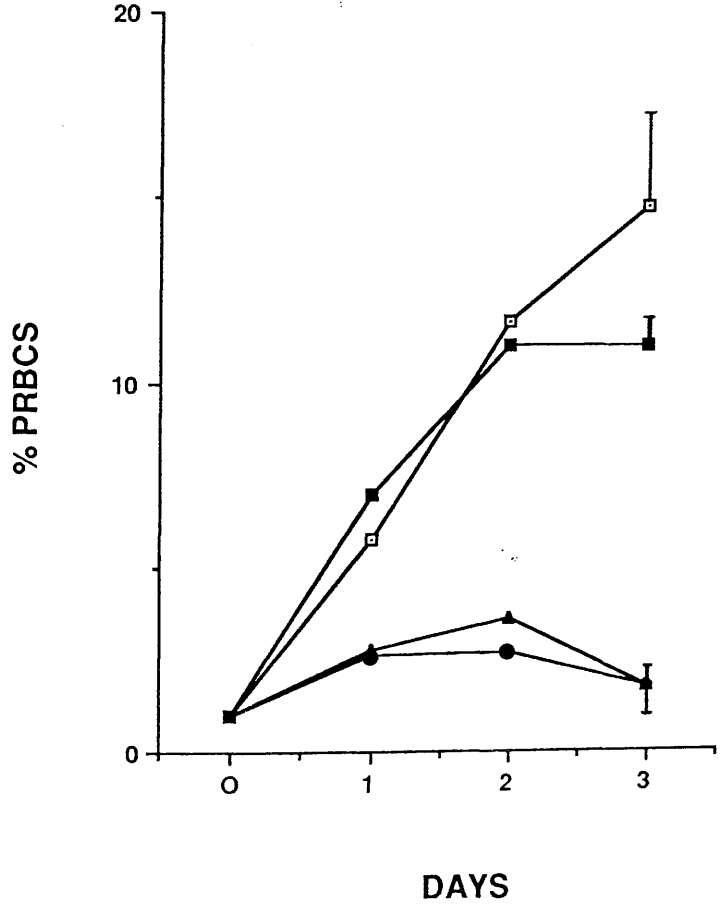
A 24 well plate was prepared as described above and in addition 3 culture plate inserts containing 500 ul of a PRBC suspension at a 10% haematocrit were placed in wells containing 1 x 10^6 PWCS in 500 µl of complete medium. Isolate RB was used with a starting parasitaemia of 1.0%. 450 µl of medium from each well and culture plate insert were replaced daily.

Result

Cultures in which inserts had been placed showed less parasite multiplication than control cultures (no inserts or PWCS) or cultures with PWCS (see Figure 3.10).

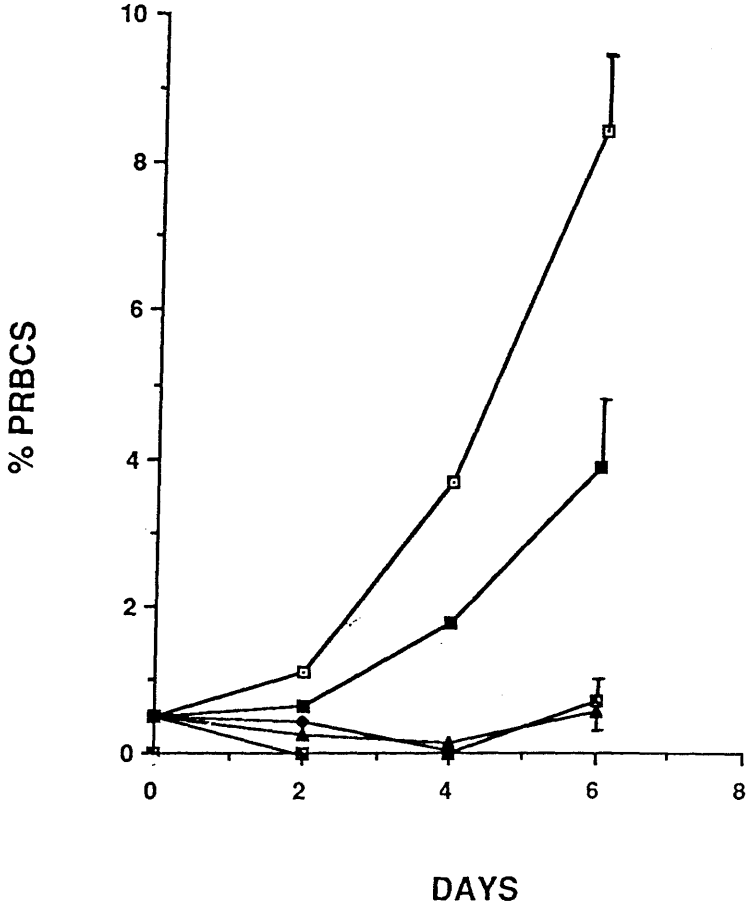
On day 6, parasite cultures grown inside culture plate inserts reached a mean parasitaemia of 0.7% which was slightly higher than cultures with inserts, either with or without PWCS which reached mean parasitaemias of 0.05% and 0.056% respectively. Control cultures (no inserts or PWCS) reached a mean parasitaemia

FIGURE 3.9 THE CULTIVATION OF ISOLATE FMG WITH PWCS AND MILLICELL H-A CULTURE PLATE INSERTS.



Isolate FMG was grown at a 10% haematocrit in 500 µl of complete medium in a 24 well multiwell plate with 1×10^6 PWCS/well. The PWCS were either in direct contact with ⊡ or were separated from the PRBCS ● by Millicell culture plate inserts. Isolate FMG was also grown without PWC in wells with ▲ or without ■ Millicell culture plate inserts. Each treatment was carried out in triplicate. Bars indicate spread of points.

FIGURE 3.10 THE CULTIVATION OF ISOLATE BG WITH PWCS AND MILLICELL H-A CULTURE PLATE INSERTS.



Isolate BG was grown at a 10% haematocrit in 500 µl aliquots of complete medium in the wells of 24 well multiwell plate ▪ or with PRBCS and $1x10^6$ PWCS in wells ▫ , or with inserts, with ◆ or without PWCS ▲ . Isolate BG was also grown inside culture plate inserts placed in wells containing PWCS ▫ . Bars indicate spread of points.

of 3.9% on day 6 and cultures containing PWCS without inserts reached a mean parasitaemia of 8.5%.

Growth of P. falciparum with Normal and Irradiated PWCS

Experiment 3.6

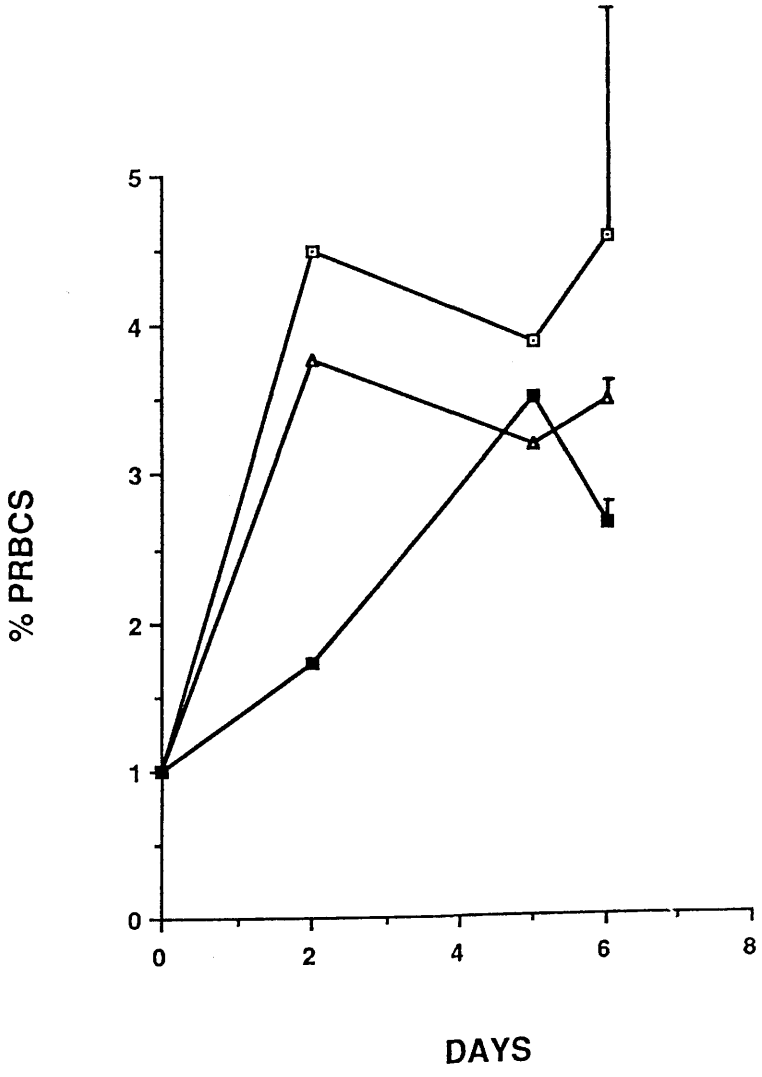
To examine the effect of disruption of PWC metabolism on the ability of these cells to promote increased parasite multiplication isolate BG was grown at a 10% haematocrit in wells containing 1×10^6 normal or irradiated PWCS in 500 µl of complete medium in a 24 well microtitre plate. BG was also grown under the same conditions without PWCS and each treatment was carried out in triplicate.

The starting parasitaemia of BG was 1.0% and 450 µl of medium from each well was replaced daily.

Result

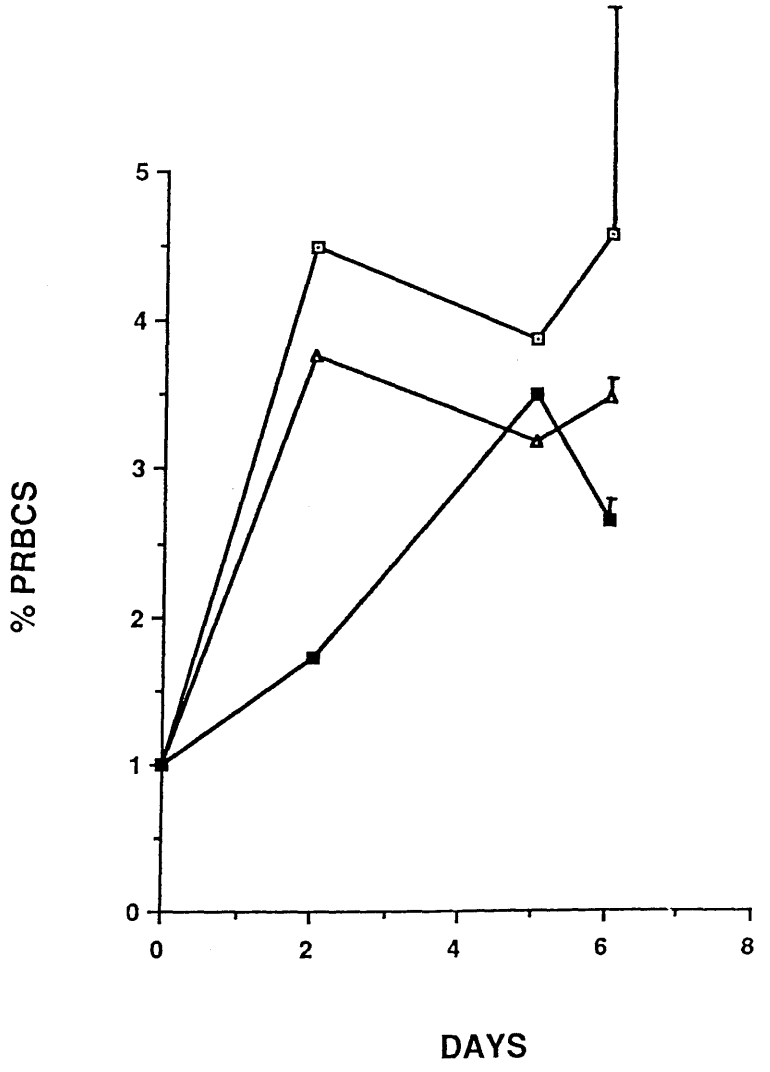
The irradiated PWCS were not as effective as normal PWCS in promoting an increase in parasite multiplication (see Figure 3.11). Cultures with irradiated PWCS reached a mean parasitaemia of 3.4% on day 6 compared with 4.5% in cultures with normal PWCS. Irradiated PWCS did, however, support more multiplication than cultures without PWCS which only reached a mean parasitaemia of 2.7% on day 6.

FIGURE 3.11 THE EFFECT OF NORMAL AND IRRADIATED PWCS ON THE GROWTH OF P. FALCIPARIM.



Isolate BG was grown at a 10% haematocrit in 500 µl aliquots of complete medium ■ and also with $1x10^6$ normal ■ or irradiated △ PWCS in a 24 well multiwell plate. Each treatment was carried out in triplicate. Bars indicate spread of points.

FIGURE 3.11 THE EFFECT OF NORMAL AND IRRADIATED PWCS ON THE GROWTH OF P. FALCIPARIM.



Isolate BG was grown at a 10% haematocrit in 500 µl aliquots of complete medium ■ and also with $1x10^6$ normal ⊡ or irradiated △ PWCS in a 24 well multiwell plate. Each treatment was carried out in triplicate. Bars indicate spread of points.

Comparison of the ability of PWCS from five strains of mice to promote increased multiplication of P. falciparum

Experiment 3.7

PWCS were routinely obtained from NIH mice as they were of the strain which was most readily available. This experiment was carried out in order to determine the suitability of PWCS from other strains of mice as feeder cells for cultures of *P. falciparum*.

PWCS were obtained from 5 strains of mice, C57 Black, DBA, C3H, NIH or BALB/C.

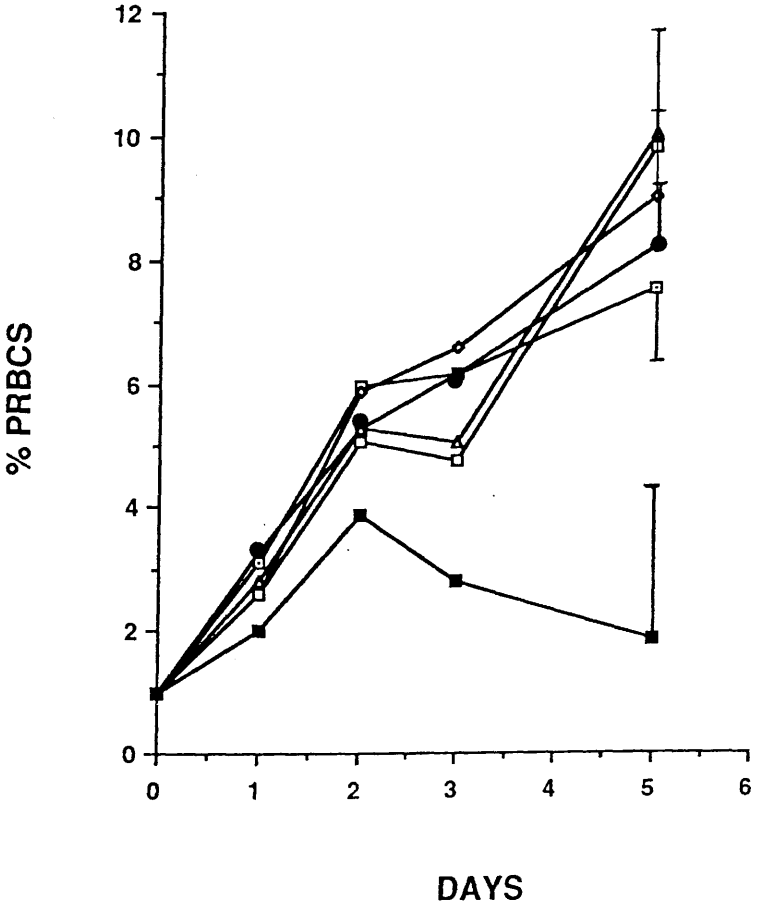
Isolate AF was grown in wells containing 1×10^6 PWCS/well, each well containing PWCS from a different strain of mouse. Parasites were grown in 700 µl aliquots of complete medium in a 24 well multiwell plate at a 3% haematocrit and also under the same conditions without PWCS. Each treatment was carried out in triplicate.

The starting parasitaemia of isolate AF was 1.0%, 500 µl of medium from each well were replaced daily and smears were made on days 1, 2, 3 and 5.

Result

PWCS from all 5 strains of mice promoted an increase in parasite multiplication (see Figure 3.12). PWCS from C3H mice promoted the greatest increase in parasite multiplication,

FIGURE 3.12 A COMPARISON OF THE ABILITY OF PWCS FROM FIVE STRAINS OF MICE TO PROMOTE AN INCREASE IN THE MULTIPLICATION OF ISOLATE AF.



Isolate AF was grow in 700µl aliquots of complete medium in a 24 well multiwell plate at a 3% haematocrit ■ . AF was also grown with $1x10^6$ PWCS from C57 Black-□ , DBA-● , C3H-△ NIH-◇ or Balb C -□ mice. Each treatment was carried out in triplicate. Bars indicate spread of points.

cultures with these cells reached a mean parasitaemia of 10.1% on day 5 compared with 1.9% in control cultures (no PWCS). PWCS from C57 Black mice promoted the smallest increase in parasite multiplication, cultures with these cells reached a mean parasitaemia of 7.6% on day 5.

The effect of the age of PWCS on the ability of these cells to promote an increase in the multiplication rate of P. falciparum.

Experiment 3.8A

This experiment was carried out in order to determine the effect of PWC age on the ability of these cells to promote an increase in the multiplication rate of P. falciparum

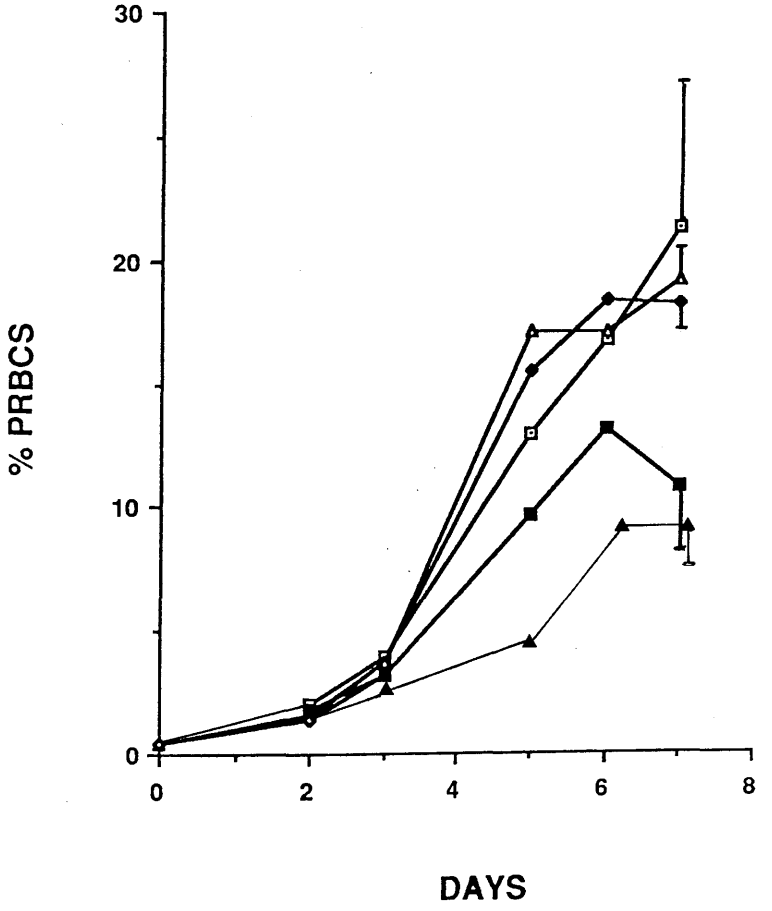
Isolate AF was grown at a 3% haemotocrit in 700 µl of complete medium in wells containing 1 x 10^6 PWCS, and also under the same conditions without PWCS, in a 24 well multiwell plate. The PWCS had been prepared 5, 3, 1 or 0 days previously and cultured in 500 µl of medium; 450 µl of which was changed daily.

The starting parasitaemia of isolate AF was 0.4% and 500 µl of medium from each well were replaced daily. Smears were made on days 2, 3, 5 and 7.

Result

All cultures containing PWCS showed increased parasite multiplication when compared with control cultures (no PWCS) (see Figure 3.13).

FIGURE 3.13 THE EFFECT OF PWC AGE ON THE ABILITY OF THESE CELLS TO PROMOTE AN INCREASE IN THE MULTIPLICATION RATE OF ISOLATE AF.



Isolate AF was grown at a 3% haematocrit in 700 µl of complete medium in a 24 well multiwell plate -▲- and also with $1x10^6$ PWCS which had been prepared 0-□-, 1-●-, 3-△- or 5-■- days previously. Prior to the experiment the PWCS had been cultured in 500 µl aliquots of complete medium in a 24 well multiwell plate, 450 µl of this medium had been changed daily. Each treatment was carried out in triplicate. Bars indicate spread of points.

Cultures with freshly prepared (day 0) PWCS had a mean parasitaemia of 21.5% on day 7. Cultures with day 1 and day 3 PWCS had mean parasitaemias of 18.4% and 19.3% respectively. This was higher than in cultures with day 5 PWCS which had a mean parasitaemia of 10.9%.

Experiment 3.8B

A 24 well multiwell plate was prepared as described above, except that prior to use the PWCS were cultured in 500 µl of complete medium and this was not changed until 24 hours after isolate AF had been added to the wells.

The experiment was maintained as described above.

Result

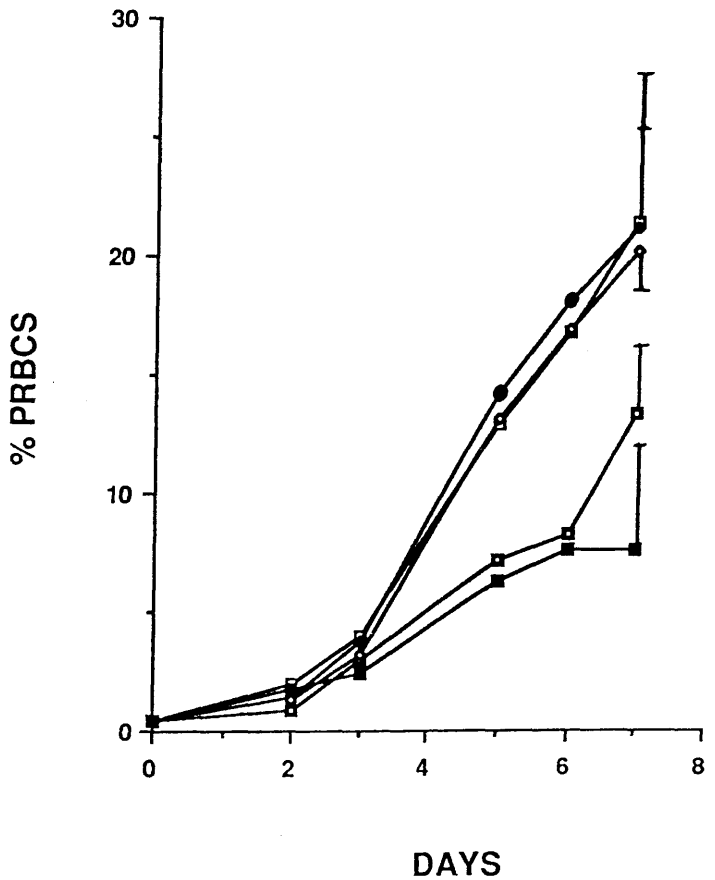
All cultures containing PWCS showed increased multiplication rates when compared with control cultures (no PWCS) (see Figure 3.14).

Cultures with freshly prepared (day 0) PWCS had a mean parasitaemia of 24.1% by day 7. Cultures with day 1 and day 5 PWCS had mean parasitaemias of 21.3% and 20.3% respectively. Cultues with day 3 PWCS had a parasitaemia of 13.4% and control cultures reached 7.7% by day 7.

Discussion

The above results show that the presence of a feeder cell layer of mouse PWCS leads to improved parasite multiplication. A

FIGURE 3.14 THE EFFECT OF PWC AGE ON THE ABILITY OF THESE CELLS TO PROMOTE AN INCREASE IN THE MULTIPLICATION RATE OF ISOLATE AF.



Isolate AF was grown at a 3% haematocrit in 700 µl of complete medium in a 24 well multiwell plate ■ and also with $1x10^6$ PWCS which had been prepared 0 ▫ , 1 ● , 3 ▪ or 5 ◇ days previously. Prior to the experiment the PWCS had been cultured in 500 µl aliquots of complete medium in a 24 well multiwell plate, this medium was not changed until 24 hours after the parasites had been added. Each treatment was carried out in triplicate. Bars indicate spread of points.

similar effect has been noted by Mazier et al. (1984) using a feeder cell layer of rodent hepatocytes co-cultured with a liver epithelial cell line. Phillips et al. (1987) have also reported the use of a feeder cell layer of human peripheral blood mononuclear cells. Neither of these methods is, however, suitable for routine application unlike the PWC method.

It has been established that the increase in parasite multiplication rate is due to an increase in the numbers of PRBCS rather than due to the destruction of non-infected RBCS by PWCS or through spontaneous lysis of RBCS in cultures. It was thought that a small decrease in the total number of RBCS/well would be observed due to the destruction of parasitised RBCS at merozoite release. This was not, however, apparent, probably due to the insensitivity of the technique used to count the total number of RBCS/well.

It is interesting to note that the effect of the PWCS on parasite proliferation was most marked with a new isolate. Not all isolates of P. falciparum can be easily adapted to in vitro growth and it would be advantageous if PWCS could be used in the adaptation of new isolates to culture; the feasibility of this is examined in chapter 4.

It appears to be the adherent (macrophage enriched) PWC population wich is most effective in promoting increased parasite growth.

It is interesting to note here that Butcher & Clancy (1984) found that monocytes from non-immune blood donors could be

significantly inhibitory to cultures of P. falciparum. These workers report that the inhibitory factor is released during the first 24 hours of monocyte culture, and it affects intracellular parasite growth. They also report that this inhibitory factor acts in a manner similar to that described by Jensen et al. (1983A) for human Sudanese sera and suggest that the molecule involved is the same in both studies. Jensen et al. (1983A) has named this molecule crisis form factor (CFF) and it may be produced by mononuclear phagocytes [Clark et al. 1981]. A variety of factors are known to be released by macrophages and monocytes [Davies & Bonney 1979]. These include tumour necrosis factor [Clark 1978], polyamine oxidase [Morgan & Christenson 1983] and free radicals [Clark & Hunt 1983] and it has been suggested that all these factors may have a role in cytotoxicity to malaria.

Taking into account the findings of Butcher and Clancy (1984) the effect of the length of time that PWCS had been cultured in vitro before being used as feeder cells was examined. There did not appear to be any advantage in culturing PWCS for any length of time before using them as feeder cells for cultures of P. falciparum. It is, however, interesting to note that when the medium was changed daily, PWCS cultured for 5 days before use supported least parasite multiplication. When medium was not replaced until 24 hours after parasites had been added to the PWC cultures it was 3 day old PWCS which were least able to support increased parasite multiplication. It would be necessary to repeat these two experiments before any conclusions could be made about these observations.

While the stimulatory effect of PWCS on cultures of P. falciparum has been noted, the underlying mechanisms which lead to increased parasite growth are not clear. It appears that the PWCS do not affect the NRBC membrane in such a way as to make it more amenable to merozoite invasion, as parasites grown in PWC conditioned RBCS did not show increased multiplication when compared with parasites grown in normal RBCS.

When cultures of P. falciparum were separated from PWCS using Millicell HA culture plate inserts no increase in parasite multiplication rate was observed. In fact the presence of the chambers with or without PWCS appeared to be inhibitory to parasite growth, suggesting that the chambers are toxic to parasites.

The fact that irradiated PWCS did not support parasite growth to the same level as non-irradiated PWCS suggests that the PWC metabolism needs to be fully functional to promote increased parasite growth as irradiation will cause a disruption of cell metabolism.

PWCS from 5 different strains of mice all promoted an increase in parasite growth. PWCS from NIH mice were used throughout this study as they were most easily available, but it would appear that any strain of mice can be used as a source of PWCS.

CHAPTER 4

The adaptation of new isolates of *P. falciparum* to continuous culture using a feeder cell layer of peritoneal wash cells

Introduction

Not all isolates of *P. falciparum* can be easily adapted to continuous *in vitro* growth and those isolates which do adapt often show poor growth during the first weeks of cultivation, [Trager and Jensen 1976, Chin and Collins 1980]. It would seem likely that parasite selection is taking place during this adaptation period, so the composition of parasite populations adapted to growth *in vitro* may differ from those in the original isolate. A number of workers have reported changes in parasite populations during *in vitro* cultivation; for example Rosario (1981), Jensen *et al.* (1981), Fenton *et al.* (1985), Ponnudura i *et al.* (1982).

This is important because it implies that the parasites cultured and studied *in vitro* may be those most able to grow under *in vitro* culture conditions rather than those best adapted for life in a host. Langreth *et al.* (1979) have shown that the *in vitro* cultivation of *P. falciparum* can lead to the loss of knob structures from the surface of infected erythrocytes. This loss of knob structures has been shown to correlate with the loss of the property of cytoadherence to human endothelial cells [Udeinya *et al.* 1983] or to amelanotic melanoma cells [Schmidt *et al.* 1982]. These K^- variants were found to be less virulent than the K+ variants in intact *Aotus* monkeys [Lanners and Trager 1984, Green *et al.* 1985] but K^- parasites grow more rapidly than K+ parasites *in vitro* [Motyl and Reese 1983].

The conservation of the entire parasite population is an important aim if *P. falciparum* cultures are to be used for

biochemical analysis, for drug screening or for antigen production Mazier et al. (1984) report that they could only adapt 3 out of 9 isolates under standard culture conditions while all 9 isolates grew well in the presence of a feeder cell layer of rodent hepatocytes.

A similar effect has also been noted by Phillips et al. (1987) when using human peripheral blood mononuclear cells as feeder cells for new isolates of P. falciparum.

The possibility that PWCS may aid new isolates to adapt to culture more readily was therefore investigated.

It may be that the faster adaptation shown by some new isolates of P. falciparum in the presence of feeder cells is the result of a larger number of individual parasites in the original isolate being able to make the transition to in vitro culture. In order to explore this possibility the parasites in one new isolate were examined using a panel of 11 monoclonal antibodies in an IFAT test. Parasites from the same isolate were examined again after being in continuous culture for 141 days both with and without PWCS.

The use of PWCS in the adaptation of new isolates of P. falciparum to continuous in vitro cultivation

Experiment 4.1

New isolates of P. falciparum were cultured according to the candle jar method of Trager and Jensen (1976), at a 10%

haematocrit in 1.5 mls of complete medium in 35 mm plastic petri dishes both with and without 1.5 x 10^6 PWCS/dish. 1 ml of medium from each petri dish was replaced daily. Fresh red blood cells were added every 3 to 5 days.

A total of 13 new isolates were cultured under the above conditions.

Result

A summary of the results is shown in Table 4A. Six of the isolates used could only be established in continuous culture in the presence of PWCS. Three isolates were established in culture both with and without PWCS and 4 of the isolates could not be adapted to *in vitro* cultivation.

The 6 isolates which adapted to *in vitro* cultivation only with PWCS were AF, LU, BG, MF, MK and WS. These isolates were maintained *in vitro* for periods of between 24 and 62 days with PWCS before being cryopreserved as stabilate or being discarded due to contamination. All the cultures contained healthy parasites when they were discontinued and all stabilates were found to be viable when recovered. Parasites were observed in cultures of these 6 isolates without PWCS for up to 22 days after cultures were initiated, depending on the isolate. Cultures were maintained for a further 7 days after the last parasite was seen before being discarded. Table 4.B shows the parasitaemias of two isolates which only adapted to *in vitro* cultivation in the presence of PWCS, during the first 24 days of cultivation.

Table 4A

The Growth of New Isolates of P. falciparum with and without PWCS

Isolate	% Parasitaemia	Origin	Treatment	Days in continuous culture +PWC	Days in continuous culture -PWC
FF	0.4	Nigeria		74	74
RC	12.4%	-		141 (st)	141 (st)
RB	1.0%	Ghana		52 (c)	43
JK	0.11%	Cameroon	Chloroquine	6	0
JMc	1.1%	Gambia		7	1
JM	1.5%	N.E. Uganda	Quinine	5	5
ML	0.0%	Kenya	Quinine Fansidar	0	0
AF	0.5	Pakistan		25 (st)	18
LU	0.36%	N. Nigeria		55 (st)	0
BG	0.6%	Tanzania		62 (st)	13
MF	0.37	-		37 (c)	22
MK	0.4%	Uganda		24 (st)	9
WS	0.4	Cameroon		27 (c)	8

st culture frozen as stabilate

c contaminated

- unknown

Table 4B

Adaptation of two isolates of P. falciparum to continuous culture using PWCS

	Isolate LU		Isolate MK	
	% PRBC		% PRBC	
Days in Culture	+ PWC	-PWC	+PWC	-PWC
0	0.36	0.36	0.4	0.4
3	0	0	1.0	0.4
6	0.05	0	0.9	0.1
7	0.16	0	1.1	0.3
9	-	-	1.8	0.1
10	0.41	0	1.0	0
12	0.9	Abandoned	-	-
14	1.3		-	-
15	-		0.9	0
17	2.0		2.0	0
19	3.1		1.2	Abandoned
21	2.5		2.2	
24	2.5		2.7	

- Blood smear not taken.

Isolates FF and RC were maintained for 74 and 141 days respectively both with and without PWCS before being cryopreserved as stabilates. Isolate RB was maintained *in vitro* with PWCS for 52 days before the culture became contaminated and was discarded and for 43 days without PWCS. All 3 of these isolates showed higher multiplication rates when cultured with PWCs than when cultured without.

Four isolates could not be established in continuous culture with or without PWCS. These isolates were, JK, JMC, JM and ML. It was, however, known that at least 3 of these patients had been given antimalarial drugs before a blood sample was taken. Which could account for their failure to thrive.

Examination of the changes occuring in a parasite population during in vitro cultivation with and without PWCs using an indirect immunofluorescence test.

Experiment 4:2

Isolate RC was received with a parasitaemia of 12.4%, the parasites were cultures *in vitro* until they had reached the schizont stage when they were used to prepare antigen slides.

Cultures of isolate RC were maintained both with and without PWCS for a period of 141 days. Antigen slides were prepared from the cultures at regular intervals.

Antigen slides prepared from the original isolate and from cultures maintained for 141 days were examined using 11 monoclonal

Table 4C

The Results of an IFAT test (carried out on 3 separate occasions) on an infected blood sample and on parasites from the same isolate (RC) after 141 days in continuous culture with and without PWCS

Monoclonal Antibody	Original Isolate			+ PWC			- PWC		
13.1.2	++	++	+++	+++	++	++	+/-	-	-
12.1-54	++	+++	++	++	+	+	+/-	-	+/-
9.2142	+++	++	++	+/-	-	-	-	-	+/-
7.3.7	-	-	+/-	-	-	+/-	-	-	+/-
13.2-3	++	+++	+++	+++	+++	+++	+++	+++	+++
9.8.441	+++	+++	+++	+++	+++	+++	+++	+++	+++
5.1-4	++	++	++	++	++	++	+	+	+
9.2-62	+++	++	++	+++	+++	+++	+++	+++	+++
9.5-15	+	++	++	+	+	+	+	+	+
12.512	++	++	++	++	+	+	++	+	+
13.421	+	++	++	++	++	++	++	+	+
-ve Control	-	-	+/-	_	-	-	-	-	-

(+++, ++, +) relative intensity of positive staining

(-) negative reaction.

antibodies (McAbs) in an indirect immunofluorescence test. The test was carried out on 3 separate occasions.

Result

A summary of results is shown in Table 4.C. McAbs 13.1-2 and 12.1-54 (which recognise a schizont antigen of approximately 200 kDa [McBride et al. 1985]) gave positive results with parasites in the original isolate and also with parasites from cultures with PWCS. These McAbs were not, however, seen to react with parasites from cultures without PWCS.

McAb 9.2142 (which recognises a 90 kDa antigen) gave positive results with parasites from the original isolate of RC but was not seen to react with parasites which had been cultured in vitro with or without PWCS for 141 days.

Discussion

These results demonstrate that the presence of a feeder cell layer of PWCS allows some new isolates of P. falciparum to be established in vitro more readily. These findings regarding the use of feeder cells in the adaptation of new isolates to culture are also in agreement with those of Mazier et al (1984) and Phillips et al. (1987).

Six of the 13 isolates used could only be maintained in vitro in the presence of PWCS. Parasitaemias achieved by 2 of these isolates (LU and MK) during the first 24 days of culture are shown in Table 4B. Isolate LU showed an initial decrease in parasite numbers when the isolate was first placed in culture. This

initial decline in parasite numbers has also been described by Trager & Jenson (1976) and Chin & Collins (1980). This was not, however, observed with isolate MK which showed an increasing parasitaemia from the first day it was placed in culture.

Four isolates could not be established *in vitro* with or without PWCS. In the case of 3 of these isolates (JK, JM and ML) the patients had been given antimalarial drugs before a blood sample was taken. Parasites observed in these isolates did not undergo reinvasion; it would seem likely that they had been affected by the antimalarial drugs and so were no longer viable when placed in culture.

It has been shown that some new isolates of *P. falciparum* can only be maintained *in vitro* in the presence of a feeder cell layer of PWCS and that PWCS can promote increased parasite multiplication rates. This suggests that parasites cultured in the presence of PWCS may be under less stress than those maintained under standard culture conditions and so a larger proportion of the individual parasites present in the population may be able to make the transition to *in vitro* cultivation.

Support for this view is provided by the findings of experiment 4.2. Isolate RC adapted to growth *in vitro* both with and without PWCS. However parasites cultured without PWCS appeared to lose the ability to react with McAbs 13.12 and 12.154 (which recognise antigens of approximately 200 kDa) while parasites cultured with PWCS for 141 days retained reactivity with these McAbs.

It is possible that the parasite population in the original isolate which reacted with these McAbs was not able to adapt to standard *in vitro* culture conditions and so may have been lost. Alternatively the proportion of this parasite population in the culture without PWCS may have been reduced to such a low level that they could not be detected using a standard IFAT test.

One McAb (9.2142, which recognises an antigen of 90 kDa) gave positive results with parasites in the original isolate but was not seen to react with parasites cultured with or without PWCS.

It would appear that the use of PWCS when adapting new isolates of *P. falciparum* to culture allows a larger number of the individual parasite populations present in an isolate to become established in culture than under standard culture conditions. Unfortunately it does however appear that even with the use of PWCS some parasite populations are lost during the transition to *in vitro* culture.

CHAPTER 5

The development of a technique for the cloning of *P. falciparum* (by limiting dilution) using a feeder cell layer of peritoneal wash cells.

Introduction

It is now well documented that malaria parasites show considerable variation, not only between species but also among organisms belonging to a single species [Reviewed Walliker 1983].

Carter & McGregor (1973) showed that Gambian isolates of P. falciparum often exhibited more than one electrophoretic form of one or more enzymes. Studies involving isolates from other parts of the world have shown that they also contain mixtures of enzyme types. [Sanderson et al. 1981; Thaithong et al. 1981, Hempelmann et al. 1981].

Rosario (1981) prepared clones from a single isolate of P. falciparum and showed that while the original uncloned material exhibited a mixture of several enzyme types, single clones exhibited only a single form of each enzyme. Clones prepared from other cultured isolates of P. falciparum have been shown to exhibit differences in susceptibility to antimalarials [Graves et al. 1984a, Thaithong et al. 1983, 1984, Trager et al. 1981, Kyle Webster et al. 1985), to exhibit antigenic diversity as detected by strain specific monoclonal antibodies [Thaithong et al. 1984] and to differ in their ability to produce gametocytes [Graves et al. 1984].

These workers cloned isolates which had been cultured in vitro for various periods of time before cloning. The shortest cultivation period is reported by Graves et al. (1984) who cloned isolate IMT-25 after 6 days in vitro cultivation. Rosario (1981) cloned an isolate from Thailand (T9) which had been cultured for a period of between 2 and 5 weeks. The original isolate was shown

to exhibit 2 forms of glucose phosphate isomerase (GPI-1 and GPI-2) and 2 forms of adenosine deaminase (ADA-1 and ADA-2). When the isolate was cloned, 2 to 5 weeks later, the band of enzyme activity for ADA 2 could no longer be detected and GPI-1 was found to be present in reduced amounts.

Most isolates are, however, cultured for even longer periods of time before being cloned. For example Green et al. (1984) report cloning an isolate previously maintained in culture for 30 months.

When cloning studies are carried out using isolates of P. falciparum which have previously been maintained in culture it is likely that these parasites will have undergone a selection process. Therefore the parasite population which was cloned may differ from that of the original isolate. It would obviously be an advantage if parasites could be cloned directly from an infected blood sample without first having to establish the isolate in culture.

With this aim in mind a feeder cell layer was used when cloning new and established isolates of P. falciparum by limiting dilution.

Clones produced in one such cloning experiment were examined using a panel of monoclonal antibodies in an indirect fluorescence test as described by McBride et al. (1982).

Wells and Price (1983) have developed a simple and rapid method for freezing hybridomas in 96 well microtitre plates. As the preparation of large numbers of PWCS is time consuming it

would be most convenient if this method could be adapted for use with PWCS. This would allow 96 well plates containing PWCS to be prepared in advance and stored until required for cloning isolates of P. falciparum.

There is a need for daily replacement of culture medium under standard culture conditions. Druhille et al. (1980, 1983) have found that by increasing the Hepes buffer concentration of RPMI 1640 from 25 mM to 35 mM the frequency of medium change can be reduced to once every 3 days. The feas bility of using this method when cloning isolates of P. falciparum was investigated as this would reduce the number of times the plate was handled and so reduce the risk of contamination.

Cloning of P. falciparum by limiting dilution with a feeder cell layer of PWCS

Experiment 5:1

Nine isolates of P. falciparum were cloned by limiting dilution (as described in Materials & Methods) in 96 well microtitre plates both with and without 4×10^5 PWCS/well. Cloning was attempted a total of 21 times, either directly from infected blood samples, from cryopreserved blood samples or from cultures. 150 µl of medium from each well were replaced daily and a smear from each well was made after 21 days.

Result

A summary of the results is shown in Table 5.A. Wells

Table 5A

Summary of cloning experiments carried out with and without a feeder cell layer of PWCS

Experiment	Isolate	Treatment before cloning	Number of positive wells		Day Plate Screened
			+PWC	-PWC	
1	FF	C - 37 days	14	not done	21
2	FF	C - 48 days	39/40	2/56	21
3	IT	P	3	0	16 (Contam)
4	LU	P	0	0	20
5	LU	C - 28 days	4	0	21 (Contam)
6	AF	St - 24 hrs C	-	-	(Contam)
7	BO	St - 24 hrs C	0	0	21
8	AF	St - 24 hrs C	16	0	21
9	JK	P	0	0	21
10	JK	C - 24 hrs	0	0	21
11	JK	P	0	0	21
12	LU	St - 24 hrs C	0	0	21
13	BO	St - 24 hrs C	0	0	21
14	BG	P	0	0	21
15	BG	P	0	0	21

Table 5.A (Continued)

Experiment	Isolate	Treatment before cloning	Number of positive wells		Day Plate Screened
			+PWC	-PWC	
16	JMc	P	0	0	21
17	RC	P	0	0	21
18	BG	C - 38 days	0	0	21
19	RC	St - 24 hrs C	0	0	21
20	JM	P	0	0	21

C - isolate cloned from culture

St - isolate cloned from stabilate

P - isolate cloned directly from patient

Contam. - culture contaminated.

containing parasites were found in 5 cloning experiments in which PWCS were used and once in a cloning experiment in which PWCS were not used.

Isolate FF was cloned after being in culture for 37 days and 14 positive (i.e. wells containing parasites) wells were found in the 96 well plate with PWCS, but no positive wells were found in the plate without PWCS. FF was cloned again after 48 days in culture and 39 out of 40 wells containing PWCS and 2 wells without PWCS were found to be positive.

Isolate IT was cloned directly from an infected blood sample and 3 positive wells were found in the plate with PWCS when the plate was screened after 16 days due to contamination.

Isolate LU was cloned after being in culture for 28 days and 4 positive wells were found in the plate with PWCS. Isolate AF was cloned from a cryopreserved blood sample which had been cultured for 24 hours prior to cloning 16 positive wells were found in the plate containing PWCS, and none in the plate without feeder cells.

Examination of Parasites from positive wells in cloning experiment 8 using an indirect immunofluorescence test (IFAT)

Experiment 5:2

When isolate AF was cloned (cloning experiment 8) 16 wells were found to contain parasites. These parasite populations were expanded in culture until sufficient material was available to

prepare antigen slides. Antigen slides were prepared from 14 clones, (2 cultures were lost through contamination before antigen slides could be made), and also from the original isolate. These were examined using a panel of 11 monoclonal antibodies (McAbs), (see Materials and Methods for specificities) in an IFAT test. Each clone was tested on 3 separate occasions.

Result

The fluorescence shown by the positive control (McAb 9.8) was scored as +++, and the negative control was scored as -, for each clone on each occasion it was tested. The level of fluorescence seen with each of the other McAbs was recorded according to this scale. A summary of the results obtained is shown in Table 5B. The parasites tested showed 5 different patterns of fluorescence, all unlike that shown by the parent isolate.

McAbs 7.3, 13.1 and 13.2 (which recognise schizont stage antigen of approximately 200 kDa) gave positive results with the parent isolate; 7.3 and 13.1 did not react with any of the clones tested and 13.2 reacted with only one clone.

Two McAbs 9.2-6 and 9.5 (which also recognise schizont antigen of approximately 200 kDa) did not give positive results with the parent isolate but gave strong fluorescence with 11 clones and weak fluorescence with 1 clone.

McAb 9.21 (which recognises an antigen of 90 kDa located in the infected RBC membrane) did not react with the parent population but gave strong fluorescence with 6 clones and weak fluorescence with 1 clone.

Table 5B

Summary of reactivities of isolate AF and twelve associated clones with eleven strain specific monoclonal antibodies.

clone	Monoclonal antibody										
	7.3	13.1	9.2-6	9.5	12.1	13.2	9.8	13.4	12.5	5.1	9.21
C11	-	-	+++	+++	-	-	+++	-	-	-	+++
C5	-	-	+++	+++	-	-	+++	-	-	-	+++
D2	-	-	+++	+++	-	-	+++	-	-	-	+++
G3	-	-	+++	+++	-	-	+++	-	-	-	+++
G6	-	-	+++	+++	-	-	+++	-	-	-	+++
C10	-	-	+++	+++	-	-	+++	-	-	-	

Table 5B Continued

clone	Monoclonal antibody										
	7.3	13.1	9.2-6	9.5	12.1	13.2	9.8	13.4	12.5	5.1	9.21
C9	-	-	+++	+++	-	-	+++	-	-	-	-
C7	-	-	+++	+++	-	-	+++	-	-	-	-
G12	-	-	+++	+++	-	-	+++	-	-	-	-
C6	-	-	+++	+++	-	-	+++	-	-	-	+/-
A9	-	-	-	+/-	-	+/-	+++	-	-	-	-
E7	-	-	-	-	-	-	+++	-	-	-	++
AF Original isolate	+++	+++	-	-	-	+++	+++	-	-	-	-

The effect of cryopreservation on the ability of PWCS to promote increased parasite multiplication

Experiment 5:3

In this experiment 96 well microtitre plates containing PWCS were thawed after being stored at -70°C for 4 days, 1, 3, 4, 5 or 16 weeks. The ability of these PWCS to promote an increase in parasite multiplication was compared with that of PWCS stored in liquid nitrogen and also with freshly prepared PWCS.

Isolates were grown at a 10% haemotocrit in 200 µl aliquots of complete medium in wells containing 4×10^5 cryopreserved PWCS. Isolates were also grown in 200 µl of complete medium with and without 4×10^5 fresh PWCS/well.

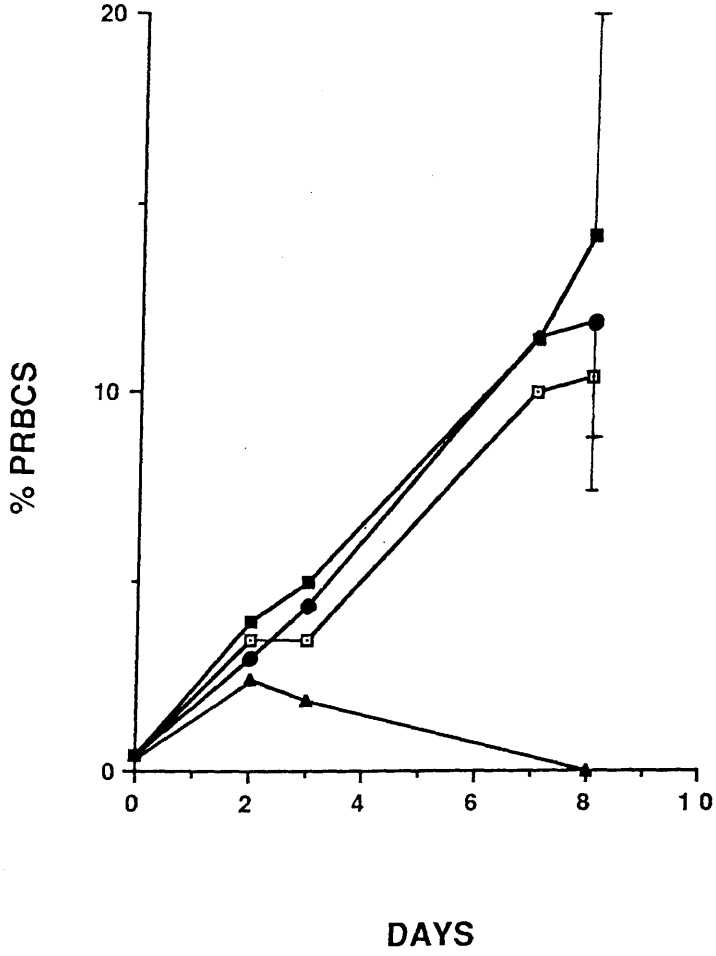
Each treatment was carried out in triplicate and 150 µl of medium from each well was replaced daily.

The experiment was carried out using isolates, AF, BG, FMG, and RB with starting parasitaemias of between 0.4% and 1.0%.

Result

After being stored at -70°C for 4 days PWCS supported parasite multiplication at a slightly lower level than fresh PWCS (see Figure 5.1). Cultures with cryopreserved PWCS had mean parasitaemias of 12.0% (frozen in FCS and 10% DMSO) and 10.0% (frozen in RPMI with 6% DMSO and FCS) on day 8 compared with 14.3% in cultures with fresh PWCS and 0% in control cultures (no PWCS).

FIGURE 5.1 THE EFFECT OF STORAGE AT -70°C FOR FOUR DAYS ON THE ABILITY OF PWCS TO PROMOTE AN INCREASE IN THE MULTIPLICATION RATE OF ISOLATE AF.



Isolate AF was grown at a 10% haematocrit in 200µl aliquots of complete medium▲ in a 96 well microtitre plate. AF was also grown under the same conditions with $4x10^5$ fresh PWCS■ or with PWCS which had been cryopreserved in FSC + 10% DMSO ● or RPMI+ 6% DMSO+FCS ⊡. Each treatment was carried out in triplicate. Bars indicate spread of points.

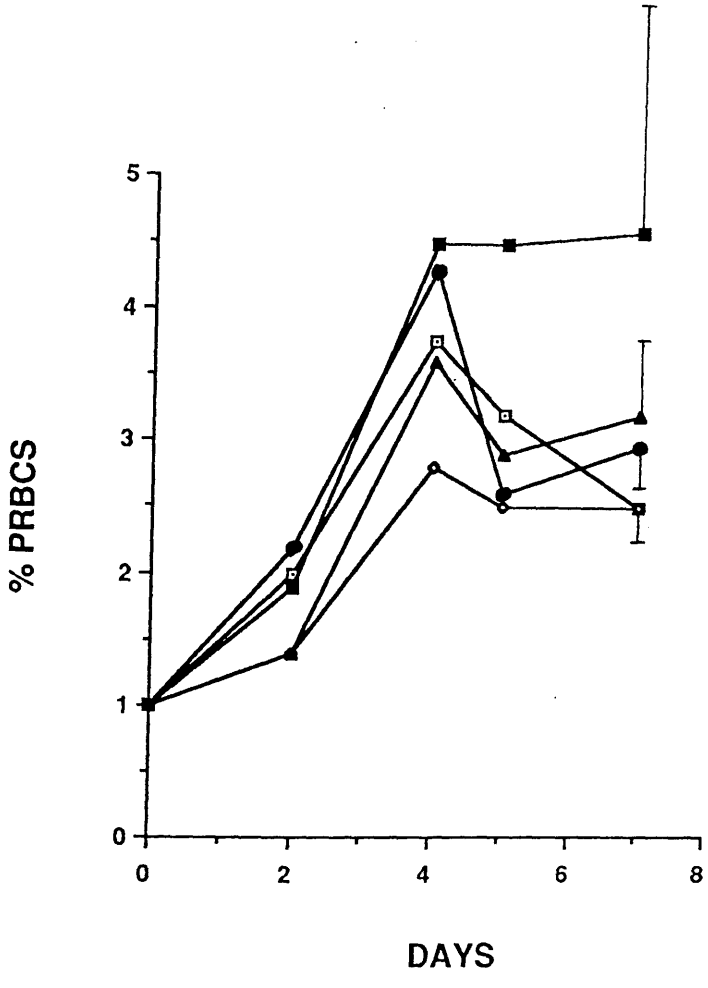
The amount of parasite multiplication promoted by PWCS after being stored at -70°C for 1 week was less than that promoted by fresh PWCS, (see Figure 5.2). Cultures with fresh PWCS achieved a mean parasitaemia of 4.6% on day 7. PWCS stored in liquid nitrogen were the most effective of the cryopreserved PWCS in promoting parasite multiplication on day 7 when these cultures reached a mean parasitaemia of 3.2%, compared with 2.5% in control cultures. On day 4, however, PWCS frozen in DMSO with 10% FCS were the most effective of the cryopreserved PWCS supporting a mean parasitaemia of 4.3% compared with 4.5% in cultures with fresh PWCS and 2.8% in control cultures.

PWCS stored at -70°C for between 3 and 5 weeks before use showed a decline in their ability to promote parasite multiplication when compared with fresh PWCS, (see Figures 5.3, 5.4 and 5.5). An exception to this were the PWCS stored in liquid nitrogen for 5 weeks, cultures with these cells had a mean parasitaemia of 6.4% on day 5 compared with cultures containing fresh PWCS at 6.3%, and control cultures at 2.4% (see Figure 5.5).

PWCS stored for 3 weeks in DMSO with 10% FCS supported a mean parasitaemia of 12.7% which compared favourably with cultures with PWCS which had a parasitaemia of 13.2% on day 5.

PWCS stored at -70°C for 16 weeks did not promote any increase in parasite multiplication (see figure 5.6).

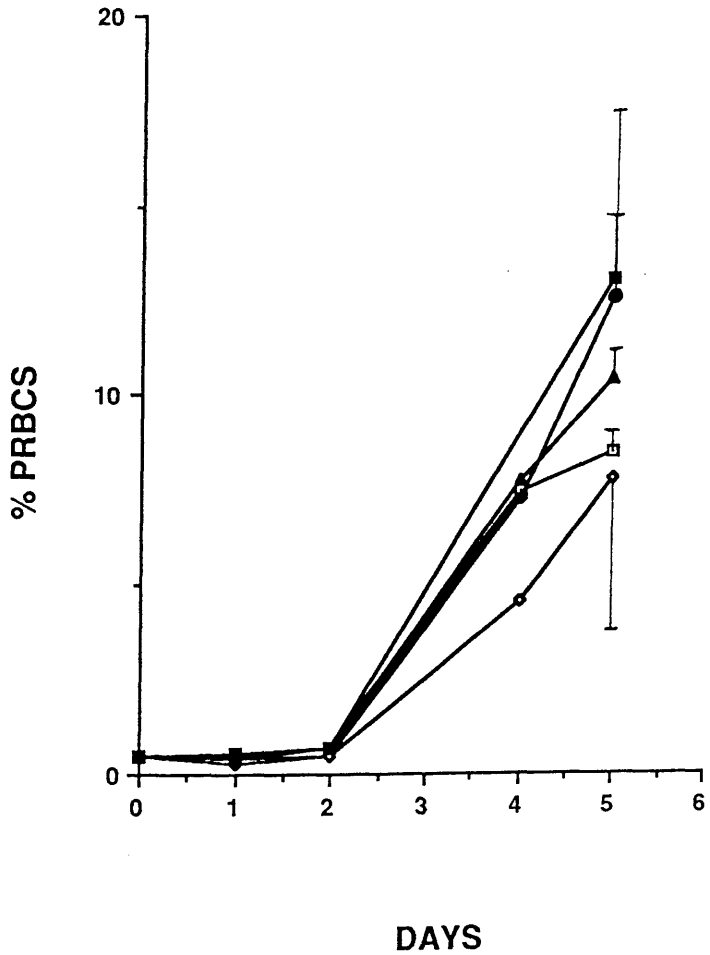
FIGURE 5.2 THE EFFECT OF STORAGE IN LIQUID NITROGEN OR AT -70°C FOR ONE WEEK ON THE ABILITY OF PWCS TO PROMOTE AN INCREASE IN THE MULTIPLICATION RATE OF ISOLATE BG.



Isolate BG was grown at a 10% haematocrit in 200 µl aliquots of complete medium -o- in a 96 well microtitre plate. AF was also grown under the same conditions with 4×10^5 fresh PWCS -■- or with PWCS which had been cryopreserved in liquid nitrogen -▲- or at -70°C using FSC + 10% DMSO -●- or at -70 C using RPMI + 6% DMSO+FCS -□- , Each treatment was carried out in triplicate. Bars indicate spread of points.

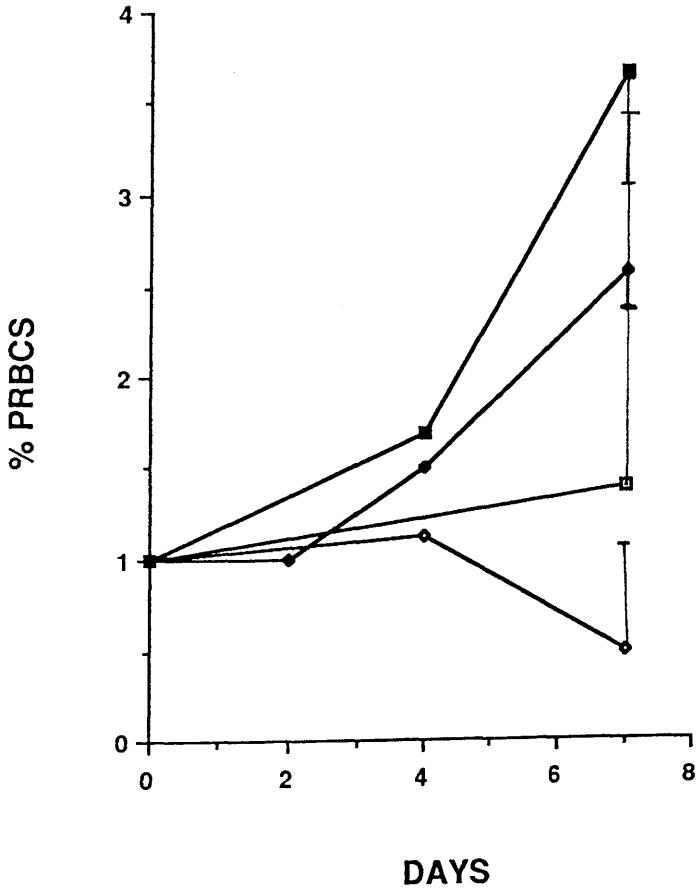
(using fcs + 10% dmso)

FIGURE 5.3 THE EFFECT OF STORAGE IN LIQUID NITROGEN OR AT -70°C FOR THREE WEEKS ON THE ABILITY OF PWCS TO PROMOTE AN INCREASE IN THE MULTIPLICATION RATE OF ISOLATE K.



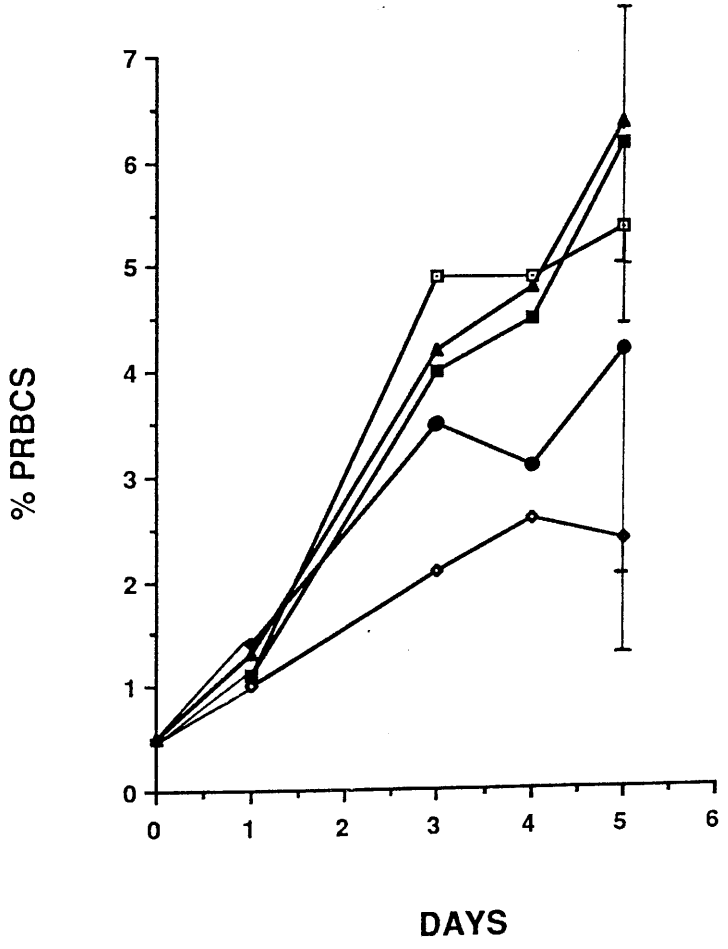
Isolate K was grown at a 10% haematocrit in 200 µl aliquots of complete medium -o- in a 96 well microtitre plate. K was also grown under the same conditions with $4x10^5$ fresh PWCS -■- or with PWCS which had been cryopreserved in liquid nitrogen -▲- or at -70°C using FSC +10% DMSO -●- or at -70 C using RPMI+ 6% DMSO+FCS -□- , Each treatment was carried out in triplicate. Bars indicate spread of points.

FIGURE 5.4 THE EFFECT OF STORAGE AT -70°C FOR FOUR WEEKS ON THE ABILITY OF PWCS TO PROMOTE AN INCREASE IN THE MULTIPLICATION RATE OF ISOLATE BG.



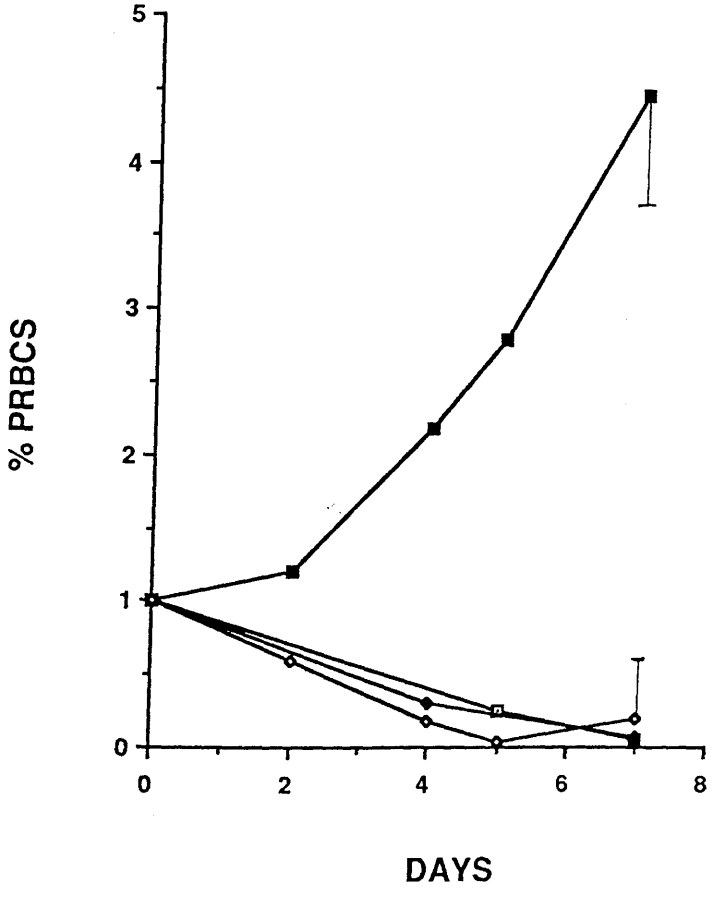
Isolate BG was grown at a 10% haematocrit in 200 µl aliquots of complete medium-◆- in a 96 well microtitre plate. AF was also grown under the same conditions with $4x10^5$ fresh PWCS-■- or with PWCS which had been cryopreserved in FSC +10% DMSO-◆- or RPMI+ 6% DMSO+FCS-□- . Each treatment was carried out in triplicate. Bars indicate spread of points.

FIGURE 5.5 THE EFFECT OF STORAGE IN LIQUID NITROGEN OR AT -70°C FOR FIVE WEEKS ON THE ABILITY OF PWCS TO PROMOTE AN INCREASE IN THE MULTIPLICATION RATE OF ISOLATE FMG.



Isolate FMG was grown at a 10% haematocrit in 200µl aliquots of complete medium -o- in a 96 well microtitre plate. FMG was also grown under the same conditions with $4x10^5$ fresh PWCS -■- or with PWCS which had been cryopreserved in liquid nitrogen -▲- or at -70°C using FSC +10% DMSO -●- or at -70 C using RPMI+ 6% DMSO+FCS -□-, Each treatment was carried out in triplicate. Bars indicate spread of points.

FIGURE 5.6 THE EFFECT OF STORAGE AT -70°C FOR FOUR MONTHS WEEKS ON THE ABILITY OF PWCS TO PROMOTE AN INCREASE IN THE MULTIPLICATION RATE OF ISOLATE RB.



Isolate RB was grown at a 10% haematocrit in 200 µl aliquots of complete medium -●- in a 96 well microtitre plate. AF was also grown under the same conditions with $4x10^5$ fresh PWCS -■- or with PWCS which had been cryopreserved in FSC +10% DMSO -◆- or RPMI+ 6% DMSO+FCS -□- . Each treatment was carried out in triplicate. Bars indicate spread of points.

The Growth of P. falciparum in RPMI containing 35 mM Hepes buffer.

Experiment 5.4A

Isolate AF was grown at a 5% haematocrit in wells containing 5×10^5 PWCS in 500µl of complete medium containing either 35 mM or 25 mM Hepes buffer in a 24 well multiwell plate. AF was also grown under the same conditions without PWCS in complete medium containing 25 mM Hepes.

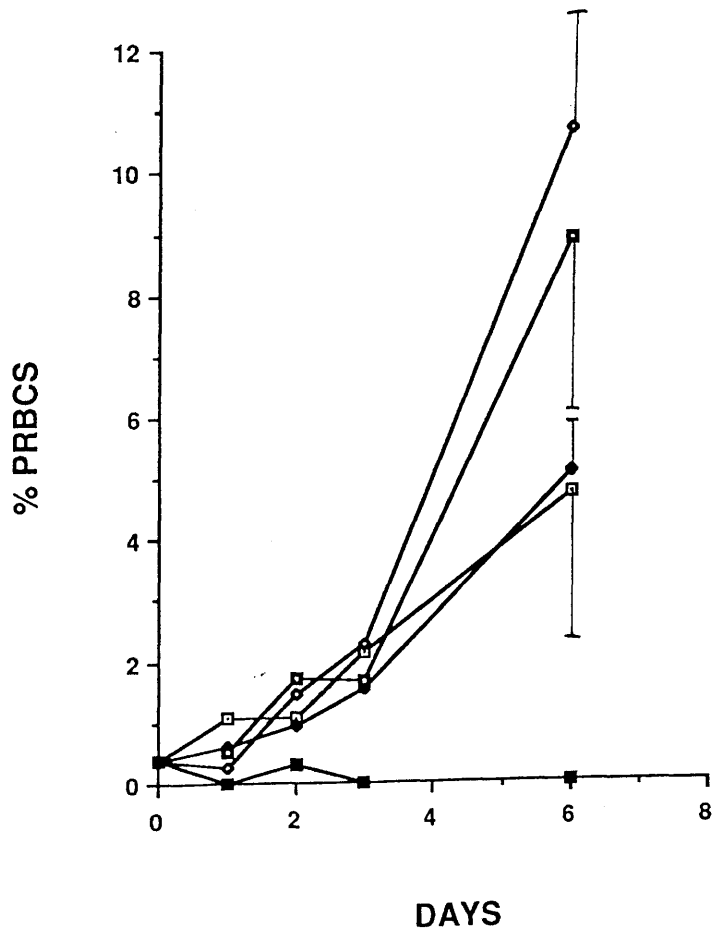
Each treatment was carried out in triplicate and 450µl of medium from each well were replaced every 24, 48 or 72 hours.

The starting parasitaemia of AF was 0.4%, the experiment was repeated using isolate AF with a starting parasitaemia of 0.5%.

Result

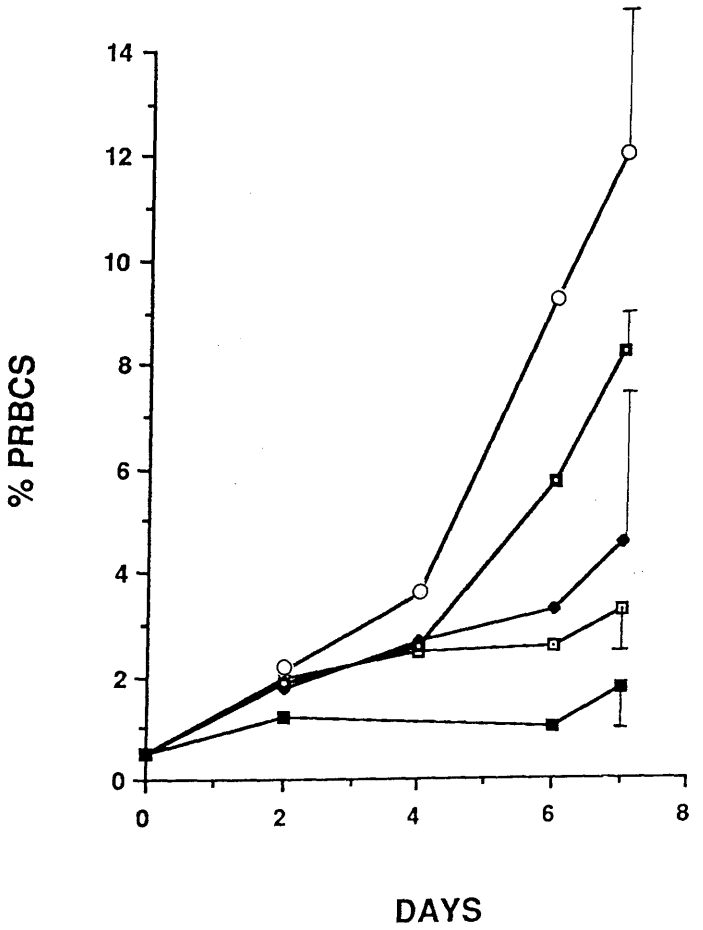
The greatest parasite multiplication was observed in cultures with PWCS and 25 mM Hepes supplemented medium which was replaced daily, (see Figure 5.7). When cultures were grown with 35 mM Hepes supplemented medium which was replaced every 24 hours they reached a mean parasitaemia of 9.0% on day 6. Cultures in which the medium was replaced every 48 or 72 hours reached mean parasitaemias of 5.2% and 4.8% respectively. All these cultures supported more parasite multiplication than control cultures (no PWCS and normal medium). A similar result was obtained when the experiment was repeated (see Figure 5.8).

FIGURE 5.7 THE EFFECT OF COMPLETE MEDIUM CONTAINING 35mM HEPES BUFFER AND REDUCED FREQUENCY OF MEDIUM CHANGE ON THE MULTIPLICATION OF ISOLATE AF.



Isolate AF was grown at a 5% haematocrit in 500 µl of complete medium containing 25mM Hepes [normal medium]■ in a 24 well multiwell plate, this medium was replaced daily. AF was also grown under the same conditions with $1x10^6$ PWCS ◇ . AF was also grown with PWCS in medium containing 35mM Hepes which was replaced every 24 □ , 48 ◆ or 72 ⊡ hours. Each treatment was carried out in triplicate. Bars indicate spread of points.

FIGURE 5.8 THE EFFECT OF COMPLETE MEDIUM CONTAINING 35mM HEPES BUFFER AND REDUCED FREQUENCY OF MEDIUM CHANGE ON THE MULTIPLICATION OF ISOLATE AF.



Isolate AF was grown at a 5% haematocrit in 500 µl of complete medium containing 25mM Hepes [normal medium] -■- in a 24 well multiwell plate, this medium was replaced daily. AF was also grown under the same conditions with $1x10^6$ PWCS -○-. AF was also grown with PWCS in medium containing 35mM Hepes which was replaced every 24 -□- , 48 -◆- or 72 -◇- hours. Each treatment was carried out in triplicate. Bars indicate spread of points.

Experiment 5:4(B)

This experiment was carried out to examine the effect of 35 mM Hepes supplemented medium and reduced frequency of medium change on cultures of P. falciparum at very low starting parasitaemias.

Isolate BO was grown at a 2.5% haemotocrit in wells containing 4 x 10^5 PWCS/well in 200 µl of complete medium containing 35 mM Hepes buffer in a 96 well microtritre plate. BO was diluted with NRBCS to give 500 PRBCS/ml. 150 µl of medium from each well were replaced every 24, 48, 72, or 96 hours. After 14 days a smear was made from each well and examined for the presence of parasites.

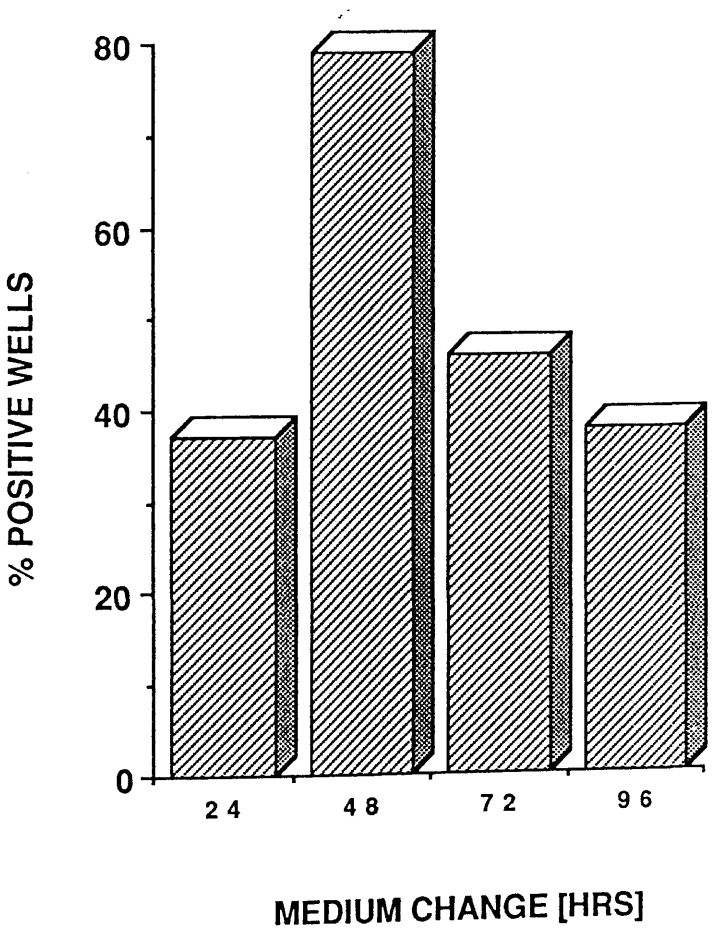
Result

37% of the wells in which the medium was replaced every 24 or 96 hours and 46% of wells in which medium was replaced every 72 hours contained parasites after 14 days. When the medium was replaced every 48 hours 79% of the wells were found to contain parasites after 14 days, (see Figure 5.9).

Discussion

The results of cloning experiments 1 to 8 are encouraging with view to cloning new isolates of P. falciparum without first having to adapt them to culture, as the presence of a feeder cell layer of PWCS appears to greatly increase cloning efficiency. In experiments 9 to 12 however, no further success was achieved,

FIGURE 5.9 THE EFFECT OF COMPLETE MEDIUM CONTAINING 35mM HEPES BUFFER AND REDUCED FREQUENCY OF MEDIUM CHANGE ON THE MULTIPLICATION OF ISOLATE BO AT A LOW STARTING PARASITAEMIA .



Isolate BO was grown at a 2.5% haematocrit and with 500 PRBCS/ml in wells containing $4x10^5$ PWCS in 200 µl of complete medium containing 35mM Hepes buffer in a 96 well microtitre plate. 150 µl of medium from each well were replaced every 24 , 48 , 72 or 96 hours.

though experiments 9 to 11, 16 and 21 were carried out on isolates which were found subsequently not to adapt to culture.

It is interesting to note that in experiments 1 to 8 RPMI from Gibco was used, while RPMI from Northumbria Biologicals Ltd., (NBL) was used in experiments 9 to 21. Initially parasite cultures appeared to grow equally well in both media, but after several months it became apparent that RPMI from NBL was less able than Gibco RPMI to support parasite growth. It is possible that parasites cultured in NBL RPMI were stressed but in standard cultures this was not immediately notic able. The stress may however, have been sufficient to noticably affect the growth of individual parasites, as would be found in cloning experiments.

There do not appear to have been any other cloning studies in which cloning was carried out directly from an infected blood sample. The shortest cultivation period is that reported by Graves et al. (1984) who cloned an isolate after 6 days in vitro cultivation. The evidence provided by other workers, such as Rosario (1981) indicates that it is desirable to clone new isolates as quickly as possible.

Isolate AF was successfully cloned from a cryopreserved blood sample which had been cultured for 24 hours prior to cloning (to check parasite viability). A total of 16 clones were obtained and the antigenic diversity of 14 of these clones was examined using strain specific monoclonal antibodies in indirect immunofluorescence tests, as described by McBride et al. (1982). These monoclonal antibodies indicated that the original isolate (AF) was a mixture of different antigenic types and in different clones ~~three~~ different antigenic types could be recognised.

Three monoclonal antibodies, 7.3, 13.1 and 13.2; which recognise a schizont stage antigen of approximately 200 kDa, gave positive results with the original isolate but did not react with any of the cloned parasite populations. This is an indication that some parasite populations present in the original isolate have not been cloned. Two monoclonal antibodies 9.2.6 and 9.5 which recognise schizont antigens of approximately 200 kDa and one monoclonal antibody recognising a 90 kDa antigen did not react with parasites in the original isolate, but did give positive results with some of the cloned parasites. A possible explanation of this may be that the parasite population expressing the responsible antigens was present in the original isolate at very low frequencies and could not be detected using a standard IFAT test.

Thaithong *et al.* (1984) also used the monoclonal antibodies of McBride *et al.* 1982 to examine 10 clones produced from an isolate from Thailand and found that at least 5 different combinations of antigen markers could be recognised. These workers also examined their clones with regard to; electrophoretic variants of 3 enzymes GPI, ADA and PEP; susceptibility to chloroquine and pyrimethimine, the presence or absence of knobs on infected erythrocytes and the 2-dimensional PAGE variants of 7 proteins. Using all these methods at least 7 different types of clone were found, this illustrates the large amount of phenotypic variation which can be observed within a single isolate. It was not reported how long this isolate had been cultured *in vitro* before cloning was carried out.

The culture supernatants of isolate AF and of the 16 clones produced from this isolate were examined with regard to S antigens. Culture supernatants from the original isolate and from 15 of the 16 cloned lines were found to contain a heat stable antigen of 145 - 155 kDa while in 1 cloned line this was approximately 10 kDa larger, (Bazarga, personal communication). This shows that the diversity of isolate AF with regard to S antigens is considerably less than demonstrated by monoclonal antibodies.

It would be interesting to examine the diversity of isolate AF and its associated clones by other methods, such as those described by Thaithong et al. (1984).

The results obtained from experiment 5:3 are disappointing as the ability of cryopreserved PWCS to promote increased parasite growth to the same level as fresh PWCS appears to decline quickly. PWC viability was not checked during this experiment but it would seem likely that the observed decline in the ability of cryopreserved PWCS to support parasite growth is due to a decrease in PWC viability. PWCs cryopreserved and stored in liquid nitrogen retained their ability to promote increased parasite multiplication a little better than PWCS frozen in 96 well plates after 5 weeks of storage. However none of the cryopreserved PWCS was able to promote increased parasite multiplication after being stored for 16 weeks.

In contrast to the findings of Druhille et al. (1980, 1983) medium containing 35 mM Hepes buffer appeared to be less able than normal medium (25 mM Hepes) to support parasite cultures at high

parasitaemias even when the medium was replaced daily. Cultures grown with medium containing 35 mM Hepes buffer did, however, achieve higher parasitaemias than control cultures (normal medium, no PWCS). The results of experiment 5:4(B) suggest that this modified medium may be suitable for maintaining cultures with reduced frequency of medium change at low parasite densities, as when the medium was replenished at 48 hr intervals 79% of wells were found to contain parasites.

CHAPTER 6

The evaluation of 4 mammalian cell lines as feeder cells for cultures of *P. falciparum*

Introduction

The use of mouse PWCS as feeder cells for cultures of P. falciparum depends upon the availability of mice as donors of PWCS and this has certain disadvantages.

It is unlikely that mouse colonies are available in many areas of the world where malaria is endemic. There is also a possibility that the PWC suspension may become contaminated as it is being removed from the mouse and that this contamination may be introduced into the parasite cultures. It would, therefore, be simpler and more convenient if a feeder cell which grows continually in culture could be found and used as an alternative to PWCS. An ideal feeder cell should be easily maintained in vitro, preferably adhere to the culture vessel and be compatable with the medium used for the cultivation of the parasites.

Cell lines which have been used as feeder cells in other systems include human fibroblasts, which were used by Michalopoulos et al. (1979) for the cultivation of parencyhymal hepatocytes and by Rheinwold and Green (1975) for the cultivation of human epidermal cells. Ishii et al. (1981) also used a human fibroblast cell line (IMR 90) as a feeder cell layer for mouse lymphoma (L1210) cells.

The use of mammalian fibroblasts as feeder cells for the cultivation of blood stream form trypanosomes is well established. Hirumi et al. (1977) used fibroblast like cells isolated from bovine blood and tissues as feeder cells for the cultivation of blood stream forms of Trypanosoma brucei in vitro. Brun et al. (1981) also successfully cultivated T. brucei, T. rhodesiense and

T. gambiense using feeder cells isolated from the embryos of New Zealand white rabbits or the mountain vole (Microtus montanus).

Three human cell lines were assessed as potential feeder cells for cultures of P. falciparum. They were selected on account of their availability and ease of cultivation. The 3 cell lines were:

Raji — A cell line derived from a Burkitts lymphoma of the maxilla and which grows in suspension.

143 — A human thymidine kinase deficient cell line derived from the mouse sarcoma virus transformed line R970-5 and which attaches to the culture vessel.

K562 — An erythroleukaemic cell line isolated from the pleural effusion of a patient with chronic myeloid leukaemia in terminal blast crisis. A proportion of the cells grow in suspension while the remainder attach to the culture vessel.

The fibroblast like cell line isolated from embryos of Microtus montanus by Brun et al. (1981) were also tested as potential feeder cells for cultures of P. falciparum.

The growth of P. falciparum with cell lines Raji and 143

Experiment 6:1A

As cell lines Raji and 143 divide in culture (unlike PWCS) they were tested as feeder cells at a lower initial concentration than that used for PWCS.

Isolate K was grown at a 3.0% haematocrit in wells containing 3.0×10^5, 1.5×10^5 or 7.5×10^4 Raji or 143 cells in 700 µl of complete medium in a 24 well multiwell plate. K parasites were also grown under the same conditions both with and without 1×10^6 PWCS/Well. Each treatment was carried out in triplicate and the starting parasitaemia of K was 1.0%. 500 µl of medium from each well were replaced daily and smears were made on days 3 and 5.

Result

All the cultures with Raji cells showed less parasite multiplication than either control cultures (no feeder cells) or cultures with PWCS (See Figures 6.1 and 6.2). This was also true for cultures with 3.0×10^5 143 cells. Cultures with 7.5×10^4 143 cells however, had a higher mean parasitaemia (5.25%) than control cultures (2.8%) on day 5. Cultures with 1.5×10^5 143 cells had a higher mean parasitaemia than control cultures on day 3 but no parasites were found in these cultures on day 5.

The parasitaemia of all cultures with Raji or 143 cells was lower than that in cultures with PWCS on both days 3 and 5.

The Growth of P. falciparum with reduced initial numbers of 143, Raji or K562 cells

Experiment 6:1B

The initial number of 143, Raji or K562 cells was reduced in this experiment as these cells appeared to multiply too rapidly in the previous experiment.

FIGURE 6.1. Isolate K was grown at a 3% haematocrit in 700 µl of complete medium in wells containing $3x10^5$ -□- , $1.5x10^5$ -●-, or $7.5x10^4$ -■- RAJI cells in a 24 well multiwell plate. K was also grown under the same conditions both with -◇- and without -▲- $1x10^6$ PWCS /well. Each treatment was carried out in triplicate. Bars indicate spread of points.

FIGURE 6.2. Isolate K was grown at a 3% haematocrit in 700 µl of complete medium in wells containing $3x10^5$ -□- , $1.5x10^5$ -●- or $7.5x10^4$ -■- 143 cells in a 24 well multiwell plate. K was also grown under the same conditions both with -◇- and without -▲- $1x10^6$ PWCS /well. Each treatment was carried out in triplicate. Bars indicate spread of points.

THE EFFECT OF CELL LINES RAJI AND 143 ON THE MULTIPLICATION RATE OF ISOLATE K.

FIGURE 6.1 CELL LINE RAJI

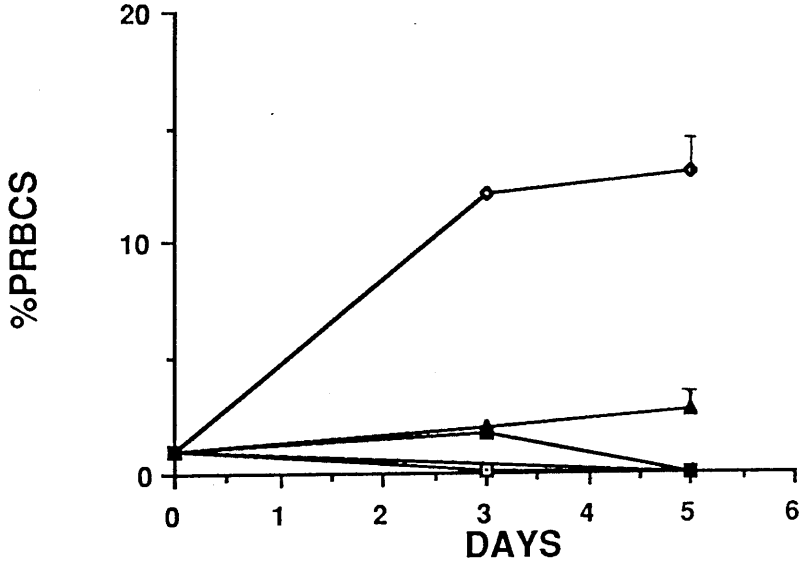
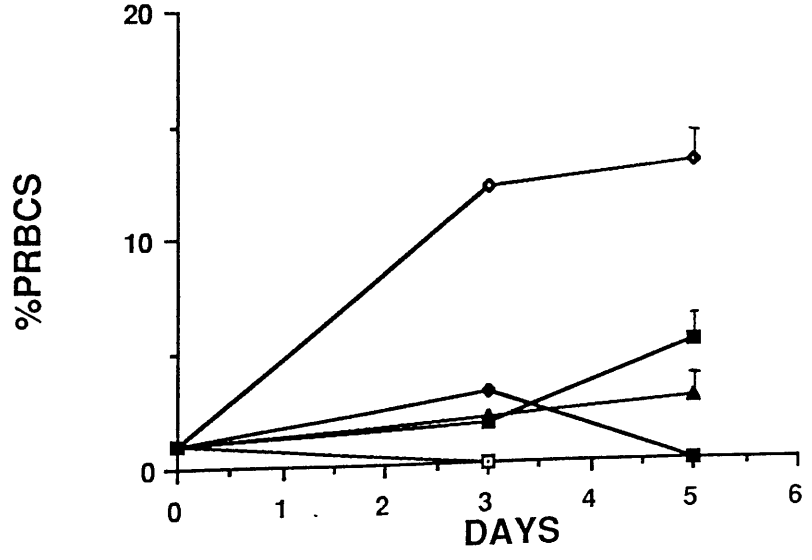


FIGURE 6.2 CELL LINE 143



Isolate K was grown at a 3.0% haemotocrit in wells containing 5×10^4, 2.5×10^4 or 1.25×10^4 Raji, 143 or K562 cells in 700 µl of complete medium in a 24 well multiwell plate. K was also grown under the same conditions in complete medium both with and without 1×10^6 PWCS/well

Each treatment was carried out in triplicate and the starting parasitaemia of K was 1.0%. 500 µl of medium from each well were replaced daily and smears made on days 3 and 5.

Result

All cultures with Raji, K562 or 143 cells had lower parasitaemias than control cultures (no feeder cells) and cultures with PWCS (see Figures 6.3, 6.4 and 6.5). On day 3, however, cultures with 1.25×10^4 Raji cells had a mean parasitaemia of 11.0% which was higher than control cultures with a mean parasitaemia of 8.4%. Cultures with PWCS achieved a mean parasitaemia of 11.7% on day 3 and 18.6% on day 5.

The growth of P. falciparum with Microtus montanus cells

Experiment 6:2

This experiment was carried out to see if M. montanus cells could be used as feeder cells for cultures of P. falciparum.

Isolates MK, RC and AF were grown at a 3.0% haemotocrit in wells containing 5×10^4, 2.5×10^4 or 1.25×10^4 M. montanus cells in 700 µl of complete medium in a 24 well multiwell plate. Isolates MK, RC and FF were also grown under the same conditions with and without 1×10^6 PWCS/Well. MK was a new isolate which had been cultured for 31 days prior to the experiment, RC and FF

FIGURE 6.3 Isolate K was grown at a 3% haematocrit in 700 µl of complete medium in wells containing $5x10^4$ ⊡ , $2.5x10^5$ ◆ or $1.25 x10^4$ ⊡ RAJI cells in a 24 well multiwell plate. K was also grown under the same conditions both with ◆ and without ▲ $1x10^6$ PWCS /well. Each treatment was carried out in triplicate. Bars indicate spread of points.

FIGURE 6.4 Isolate K was grown at a 3% haematocrit in 700 µl of complete medium in wells containing $5x10^4$ ⊡ , $2.5x10^5$ ◆ or $1.25 x10^4$ ⊡ 143 cells in a 24 well multiwell plate. K was also grown under the same conditions both with ◆ and without ▲ $1x10^6$ PWCS /well. Each treatment was carried out in triplicate. Bars indicate spread of points

FIGURE 6.5 Isolate K was grown at a 3% haematocrit in 700 µl of complete medium in wells containing $5x10^4$ ⊡ , $2.5x10^5$ ◆ or $1.25 x10^4$ ⊡ K562 cells in a 24 well multiwell plate. K was also grown under the same conditions both with ◆ and without ▲ $1x10^6$ PWCS /well. Each treatment was carried out in triplicate. Bars indicate spread of points

THE EFFECT OF CELL LINES RAJI, 143 AND K 562 ON THE MULTIPLICATION RATE OF ISOLATE K.

FIGURE 6.3 CELL LINE RAJI

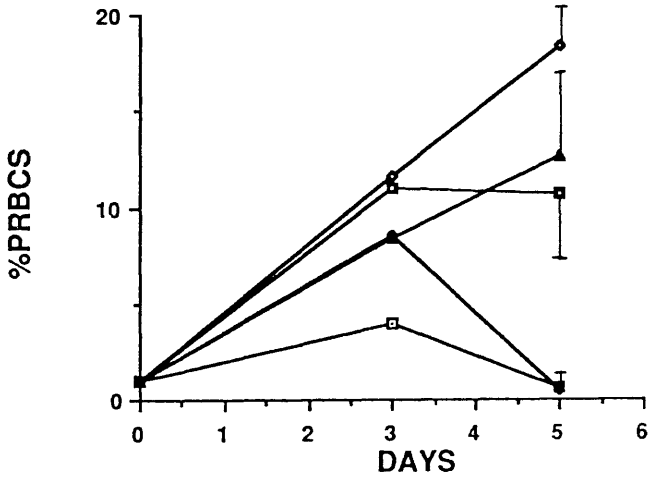


FIGURE 6.4 CELL LINE 143

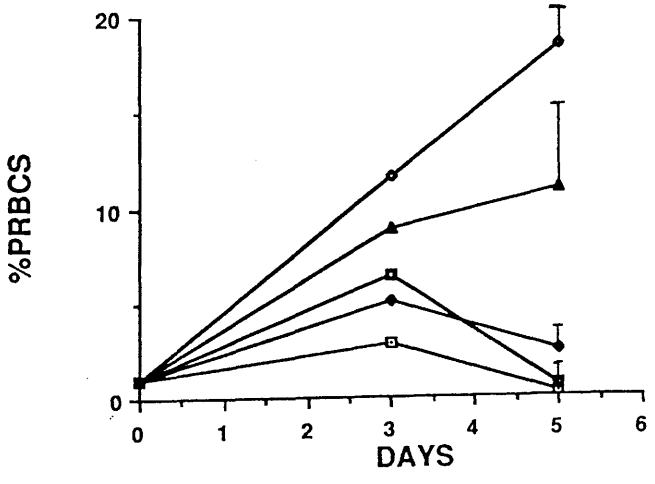
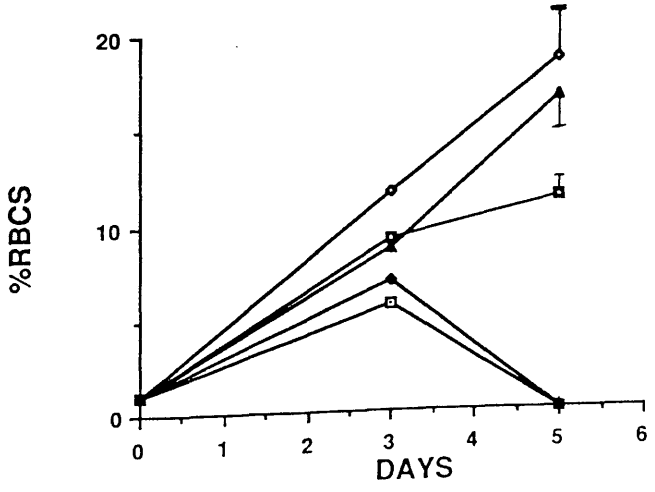


FIGURE 6.5 CELL LINE K562



had been maintained in culture for approximately 4 months prior to the experiment and were considered to be established isolates. Each treatment was carried out in triplicate and the starting parasitaemias of isolates MK, RC and AF were 1.2%, 1.5% and 1.7% respectively.

500 µl of medium from each well were replaced daily and smears were made on days 2, 3 and 5.

Results

All cultures with *M. montanus* cells showed higher parasitaemias than control cultures (no feeder cells) (see Figures 6.6, 6.7 and 6.8).

Cultures with 1×10^5 and 5×10^4 *M. montanus* cells showed the highest parasitaemias. The increase in parasite multiplication was most marked with isolate RC. Cultures of this isolate with 5×10^4 *M. montanus* cells reached a mean parasitaemia of 24.0% on day 5 compared with 23.8% in cultures with PWCS and 14.4% in control cultures.

Discussion

It has been shown that mouse PWCS can be used successfully as feeder cells in cultures of *P. falciparum*. It would nevertheless be more convenient if a suitable cell line could be found to replace PWCS and so dispense with the need to use mice as a source of feeder cells.

Three human cell lines were tested as potential feeder cells. These were chosen because they were readily available and grew

FIGURE 6.6 Isolate MK was grown at a 3% haematocrit in 700 µl of complete mediumin wells containing $1x10^5$ ⊡ , $5x10^4$ ◆ or $2.5 x10^4$ ■ M. montanus cells in a 24 well multiwell plate. MK was also grown under the same conditions both with ◆ and without ▲ $1x10^6$ PWCS/well. Bars indicate spread of points.

FIGURE 6.7 Isolate FF was grown at a 3% haematocrit in 700 µl of complete mediumin wells containing $1x10^5$ ⊡ , $5x10^4$ ◆ or $2.5 x10^4$ ■ M. montanus cells in a 24 well multiwell plate. MK was also grown under the same conditions both with ◆ and without ▲ $1x10^6$ PWCS/well. Bars indicate spread of points.

FIGURE 6.8 Isolate RC was grown at a 3% haematocrit in 700 µl of complete mediumin wells containing $1x10^5$ ⊡ , $5x10^4$ ◆ or $2.5 x10^4$ ■ M. montanus cells in a 24 well multiwell plate. MK was also grown under the same conditions both with ◆ and without ▲ $1x10^6$ PWCS/well. Bars indicate spread of points.

THE EFFECT OF *MICROTUS MONTANUS* CELLS ON THE MULTIPLICATION RATE OF THREE ISOLATES OF *P. FALCIPARUM.*

FIGURE 6.6 ISOLATE MK

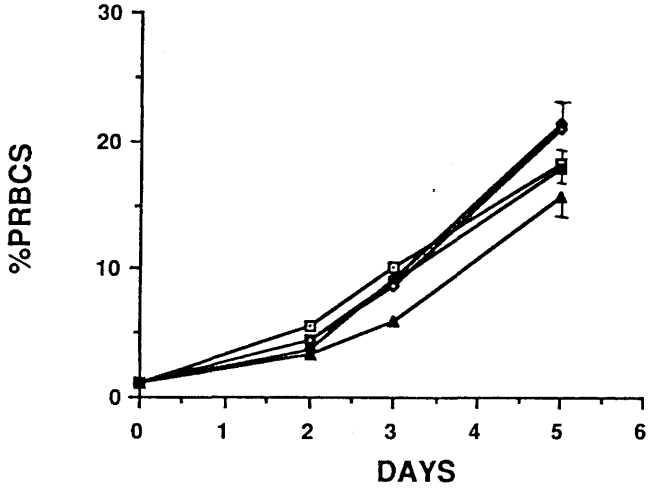


FIGURE 6.7 ISOLATE FF

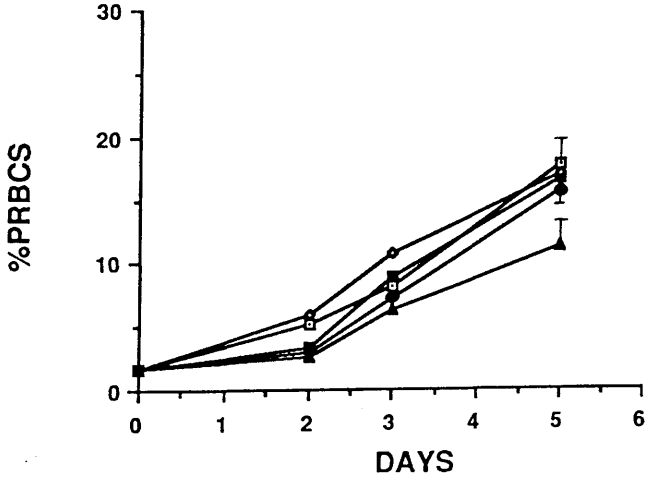
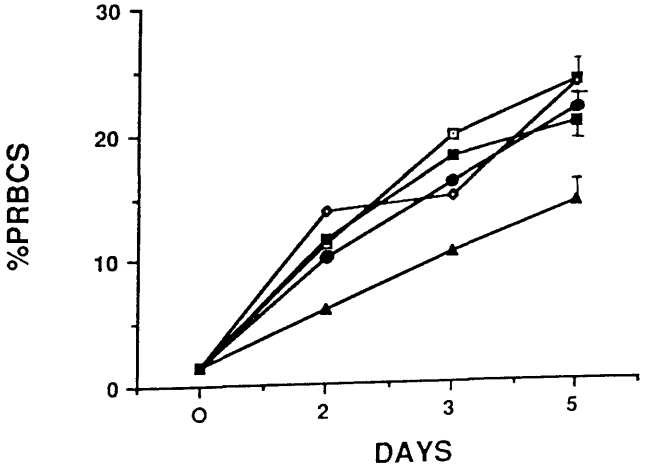


FIGURE 6.8 ISOLATE RC



well *in vitro*. K562 and 143 also attached to the culture vessel which is a desirable feature in a feeder cell. K562, 143 and Raji were not, however, effective in promoting an increase in parasite growth. Mazier *et al*. (1984) tested two human hepatoma cell lines (Hep G2 and PLC PRFS), one rat hepatoma (Faza 967), human embryonic and liver fibroblasts, and rat liver epithelial cells as feeder cells for cultures of *P. falciparum*. These cells were not able to promote an increase in parasite multiplication. From this it is apparent that not all cells are suitable for use as feeder cells when used under the conditions of this experiment.

One problem noted by Mazier *et al*. (1984) was that the cell line Hep G2 (Knowles *et al*. 1983) continued to grow rapidly even when the cells had reached confluence and so caused the culture medium to become quickly exhausted. It is likely that this was also the case with Raji, 143 and K562 cells in the experiments reported here. All *P. falciparum* cultures with these cell lines in experiment 6:1B showed an increase in parasite numbers between days 0 and 3 but parasite numbers declined sharply by day 5 suggesting that the cell lines were continuing to divide and were out growing the malaria parasites, even though the medium was changed daily. It would be interesting to repeat this work using Raji, K562 and 143 cells which had either been irradiated or chemically treated (for example with Mitomycin) to prevent them from dividing in culture.

Mazier *et al*. (1984) did successfully use a feeder cell layer of rodent hepatocytes co-cultivated with a liver epithelial cell line to promote increased multiplication of *P. falciparum* *in*

vitro. This group suggest that in their system the feeder cell effect is related to a function of fully differentiated hepatocytes as other cell types which were tested were not effective as feeder cells.

It had previously been shown [Brun et al. 1981] that Microtus montanus cells could be used successfully as feeder cells for the cultivation of bloodstream form trypanosomes.

When cultures of P. falciparum were grown in the presence of a feeder cell layer of M. montanus cells these were found to be as effective as PWCS in promoting increased parasite multiplication.

The mechanism by which feeder cells promote the growth of bloodstream trypanosomes has been investigated by Tanner (1980). This worker reports that feeder cells cannot be replaced by feeder cell conditioned medium, but that they can be replaced by medium supplemented with cysteine. This is investigated in relation to PWCS in Chapter 7.

CHAPTER 7

The effect of medium preconditioned by peritoneal wash cells or supplemented with either L-cysteine or 2-mercaptoethanol on the growth of *P. falciparum*.

Introduction

In Chapter 6, the growth of *P. falciparum* cultures with a feeder cell layer of *Microtus montanus* cells was described. This cell line, used as a feeder cell layer for the cultivation of bloodstream stage trypanosomes was found to be effective in promoting an increase in the growth of *P. falciparum*.

Since *M. montanus* cells can replace PWCS as feeder cells in cultures of *P. falciparum* it may be that the mechanism involved in promoting an increase in parasite growth is the same in the case of both cell populations.

Feeder cells have been used in the cultivation of blood stage trypanosomes for a number of years and attempts have been made to elucidate the mechanisms involved [Tanner 1980, Duszenko *et. al.* 1985].

Tanner (1980) reports that medium pre-conditioned by feeder cells was ineffective in promoting the growth of trypanosomes. The effect of PWC conditioned medium on the growth of *P. falciparum* was therefore examined.

It has been reported that the requirement of cultures of bloodstream form trypanosomes for feeder cells can be replaced by the supplementation of media with L-cysteine [Duszenko *et al.* 1985] or 2-mercaptoethanol [Baltz *et al.* 1985, Duszenko *et al.* 1985]. The effect of L-cysteine or 2-mercaptoethanol supplemented medium on cultures of *P. falciparum* was therefore examined.

Growth of P. falciparum with PWC Conditioned Medium

Experiment 7.1A

This experiment was carried out to test the effect of PWC conditioned medium on the growth of *P. falciparum*. Complete medium was conditioned by PWCS for 24, 48, 72, 96 or 120 hours. Isolate FF was grown in 700 µl aliquots of this conditioned medium in a 24 well plate at a 10% haematocrit. The conditioned medium had been stored at -20°C prior to use.

FF was also grown in 700 µl aliquots of normal medium both with and without 1 and 10^6 PWCS/Well. Each treatment was carried out in triplicate and the starting parasitaemia of FF was 0.5%. 500 µl of medium from each well were replaced daily and smears were made on days 3, 5 and 7.

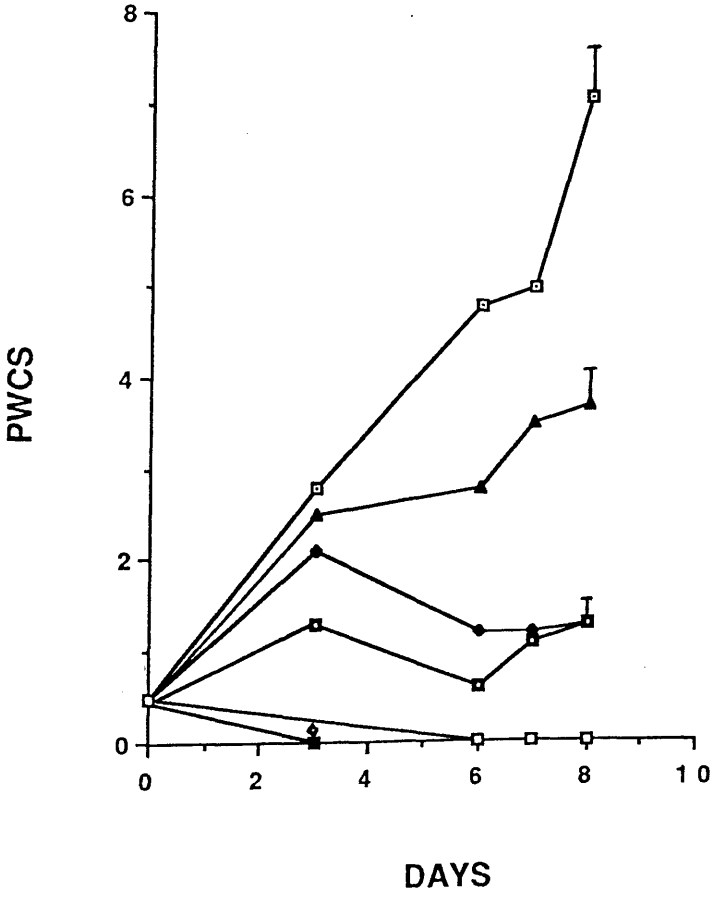
Results

No parasites were found after day 3 in cultures with 72, 96 or 120 hour PWC conditioned medium. The 24 and 48 hr conditioned medium did support some parasite multiplication; these cultures reached a mean parasitaemia of 1.3% on day 8, but this was lower than in the control cultures (normal medium, no PWCS) with a mean parasitaemia of 3.7% and cultures with PWC's at 7.1% (see Figure 7.1).

Experiment 7:1B

Isolate FF was grown under the same conditions as described above except that the conditioned medium was diluted 1:1 or 1:3 with normal complete medium.

FIGURE 7.1 THE EFFECT OF PWC CONDITIONED MEDIUM [STORED AT -20 C BEFORE USE] ON THE MULTIPLICATION RATE OF ISOLATE AF.



Isolate FF was grown at a 10% haematocrit in a 24 well multiwell plate in 700 µl aliquots of medium which had been conditioned by PWCS for 24 -◆- , 48 -■- , 72 -●- , 96 -▲- or 120 -□- hours. FF was also grown under the same conditions with normal medium both with -□- and without -▲- $1x10^6$ PWCS/ well. Each treatment was carried out in triplicate. Bars indicate spread of points.

Result

All cultures with conditioned medium supported some parasite multiplication, (see Figures 7.2 and 7.3). All these cultures, however, had lower parasitaemias by day 7 than cultures with normal medium or PWCS.

Experiment 7.1C

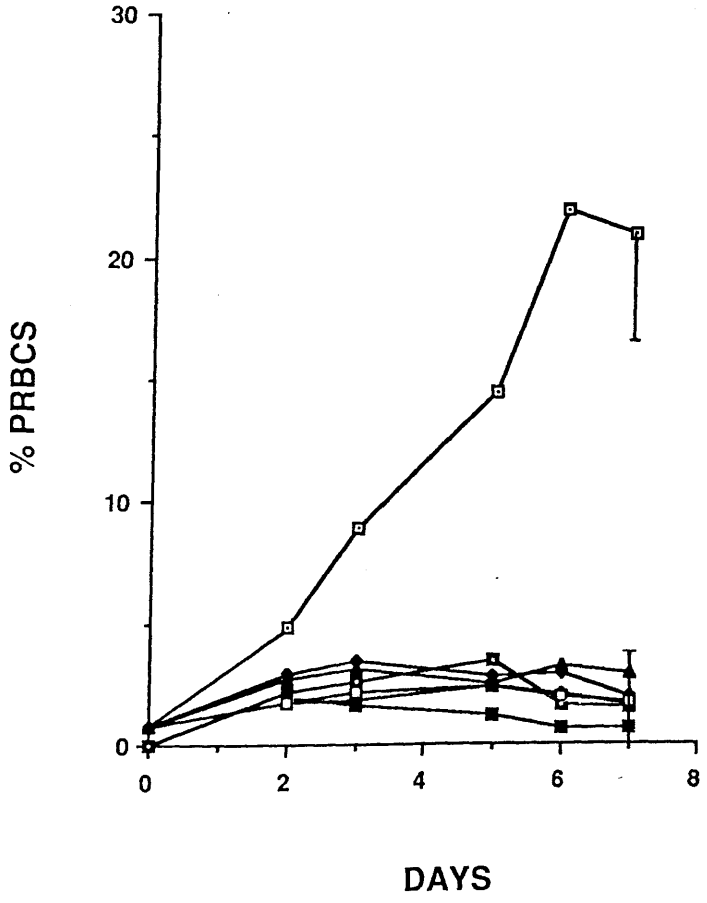
In this experiment the effect of medium conditioned by PWCS for 24 hours immediately preceeding use was tested on the growth of P. falciparum.

Isolate FF was grown at a 5% haematocrit in wells containing 500 µl of complete medium both with and without 1 and 10^6 PWCS/well. FF was also grown under the same conditions with the freshly prepared PWC conditioned medium. 450 µl of medium from each well were replaced daily and smears were made on 2, 3, 5 and 7.

Result

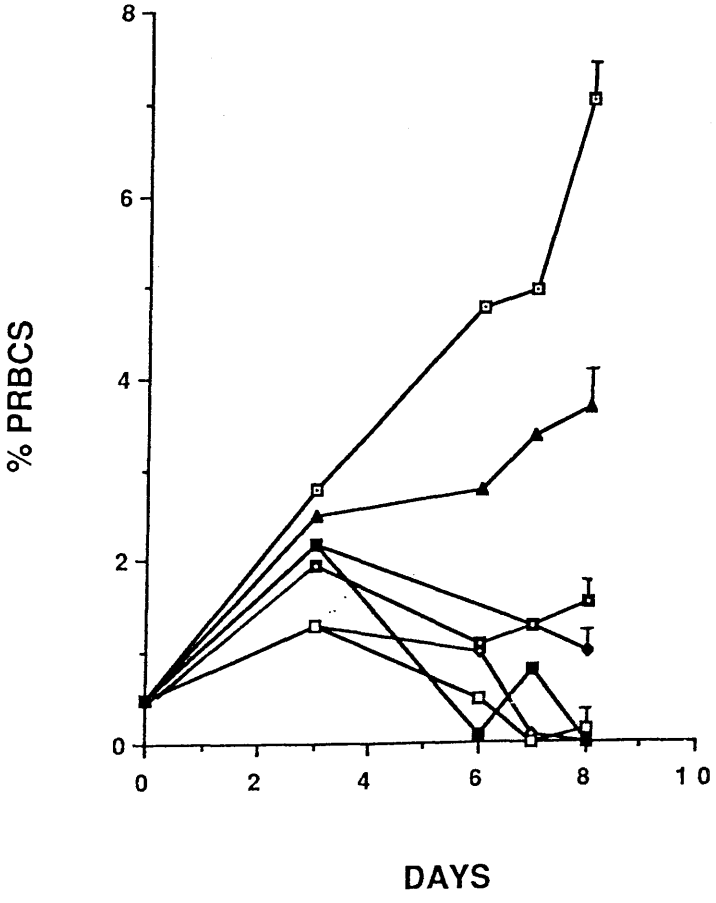
Medium conditioned by 24 hours immediately preceding use, promoted a mean parasitaemia of 6.5% on day 7 which was higher than control cultures at 3.3%, this conditioned medium was however less efective than PWCS themselves which promoted a mean parasitaemia of 9.2% on day 7 (see Figure 7.4)

FIGURE 7.2 THE EFFECT OF PWC CONDITIONED MEDIUM DILUTED 1:1 WITH NORMAL MEDIUM ON THE MULTIPLICATION RATE OF ISOLATE FF.



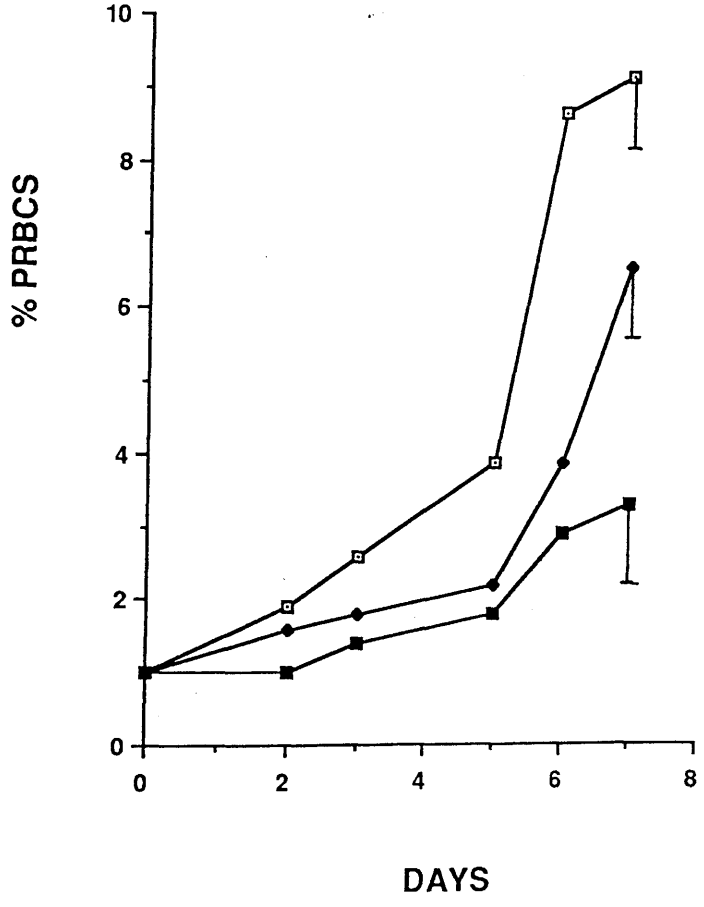
Isolate FF was grown at a 10% haematocrit in a 24 well multiwell plate in 700 µl aliquots of medium which had been conditioned by PWCS for 24 ◆ , 48 ▫ , 72 ◆ , 96 ■ or 120 ▫ hours and diluted 1:1 with normal medium. FF was also grown under the same conditions with normal medium both with ▫ and without ▲ $1x10^6$ PWCS/ well. Each treatment was carried out in triplicate. Bars indicate spread of points.

FIGURE 7.3 THE EFFECT OF PWC CONDITIONED MEDIUM DILUTED 3:1 WITH NORMAL MEDIUM ON THE MULTIPLICATION RATE OF ISOLATE FF.



Isolate FF was grown at a 10% haematocrit in a 24 well multiwell plate in 700µl aliquots of medium which had been conditioned by PWCS for 24 , 48 , 72 , 96 or 120 hours and diluted 3:1 with normal medium. FF was also grown under the same conditions with normal medium both with and without $1x10^6$ PWCS/ well. Each treatment was carried out in triplicate. Bars indicate spread of points.

FIGURE 7.4 THE EFFECT OF FRESHLY PREPARED PWC CONDITIONED MEDIUM ON THE MULTIPLICATION RATE OF ISOLATE AF.



Isolate FF was grown at a 5% haematocrit in 500 µl of complete medium both with ⊡ and without ■ $1x10^6$ PWCS/well in a 24 well multiwell plate. FF was also grown without PWCS in 500 µl of freshly prepared PWC conditioned medium. ◆
Each treatment was carried out in triplicate. Bars indicate spread of points.

The growth of P. falciparum with L. cysteine supplemented medium

Experiment 7.2A

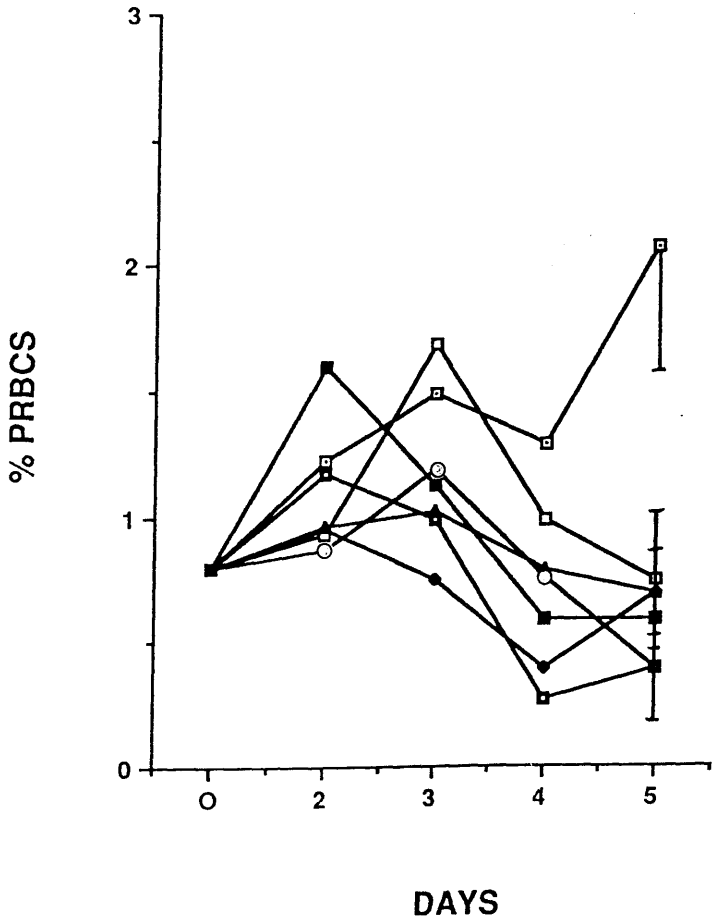
Isolate AF was grown at a 10% haemotocrit in wells containing 200 µl of complete medium supplemented with 24, 12, 6, 3 or 1.5 mg/l L-cysteine in a 96 well microtitre plate. AF was also grown under the same conditions in normal complete medium and also in medium containing 2 and 10^6 PWCS/ml. The L-cysteine supplemented medium was prepared on day 0 stored at 4^oC, and used throughout the experiment.

Each treatment was carried out in triplicate and the starting parasitaemia of AF was 0.8%. 150 µl of medium from each well were replaced every 12 hours and smears were made on days 2, 3, 4 and 5. The experiment was repeated using isolate API with a starting parasitaemia of 0.2%.

Result

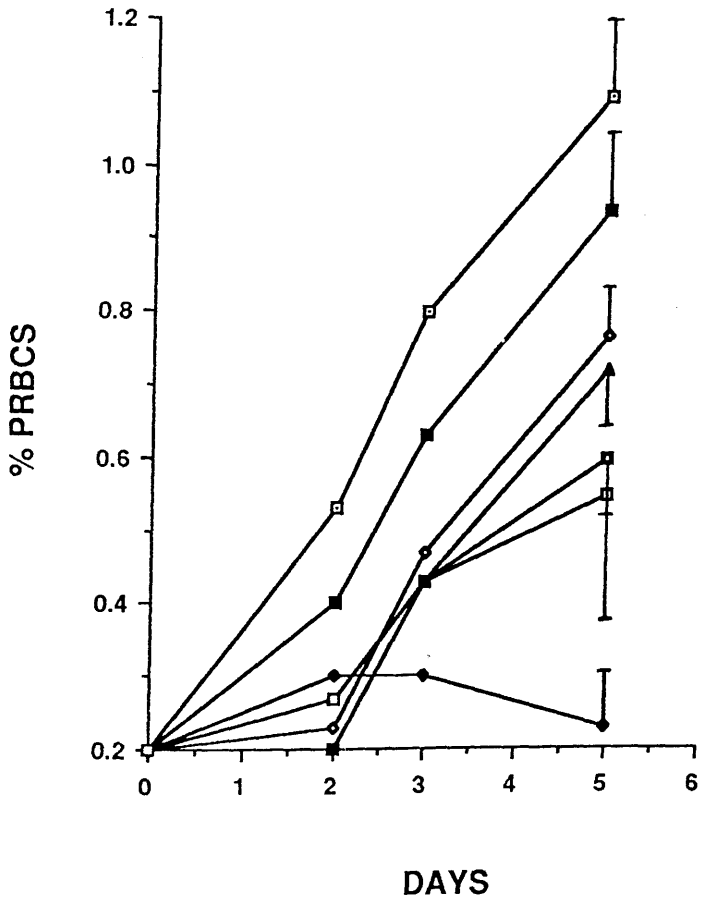
All cultures grown in medium supplemented with L-cysteine showed less parasite growth than cultures with PWCS on day 5 (see Figures 7.5 and 7.6). Media supplemented with 3 and 6 mg/l L-cysteine supported parasitaemias of 0.94% and 0.77% respectively on day 5 (Isolate AF1 Figure 7.6) which were slightly higher than control cultures at 0.72%. Only those cultures of FF grown in medium supplemented with 1.5 mg/l L-cysteine showed more parasite growth than control cultures on day 5, however, on day 2 medium supplemented with 3 mg/l L-cysteine supported a mean parasitaemia of 1.6% which was greater than cultures with PWCS at 1.2%.

FIGURE 7.5 THE CULTIVATION OF ISOLATE AF IN MEDIUM SUPPLEMENTED WITH L-CYSTEINE.



Isolate AF was grown in a 96 well microtitre plate at a 10% haematocrit in 200 µl aliquots of complete medium supplemented with 24 , 12 , 6 , 3 or 1.5 mg/l L cysteine. This medium was prepared on day 0 and used throughout the experiment. AF was also grown under the same conditions in normal medium both with and without $2x10^6$ PWCS/ml. Each treatment was carried out in triplicate. Bars indicate spread of points.

FIGURE 7.6 THE CULTIVATION OF ISOLATE AP1 IN MEDIUM SUPPLEMENTED WITH L-CYSTEINE.



Isolate AP1 was grown in a 96 well microtitre plate at a 10% haematocrit in 200 µl aliquots of complete medium supplemented with 24 ◆, 12 ■, 6 ◇, 3 ▲ or 1.5 □ mg/l L cysteine. This medium was prepared on day 0 and used throughout the experiment. AP1 was also grown under the same conditions in normal medium both with ■ and without ▲ $2x10^6$ PWCS/ml. Each treatment was carried out in triplicate. Bars indicate spread of points.

Experiment 7.2B

Isolate BG was grown at a 10% haematocrit in wells containing 200 µl of complete medium supplemented with 0.75 mg/l L-cysteine in a 96 well microtitre plate for 6 hours. The medium was then removed and replaced with medium supplemented with 24, 12, 6, 3 or 1.5 mg/l L-cysteine.

BG was also grown in normal complete medium and in medium containing 2 x 10^6 PWCS/ml.

Each treatment was carried out in triplicate and the starting parasitaemia of BG was 0.8%. 150 µl of medium from each well were replaced every 12 hours and the L-cysteine supplemented medium was freshly prepared immediately before use.

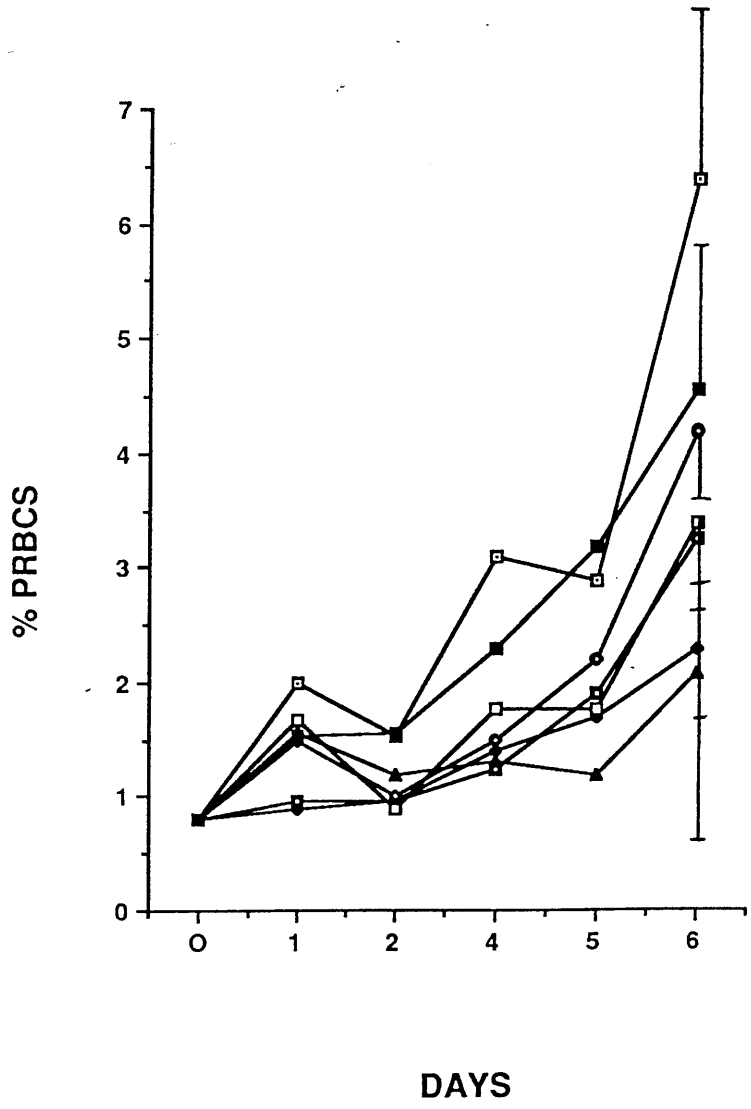
The experiment was repeated using isolate RB with a starting parasitaemia of 1.0%

Result

All cultures with L-cysteine supplemented medium supported higher parasitaemias than control cultures on day 6, except cultures of BG with 24 mg/l supplemented medium (see Figures 7.7 and 7.8).

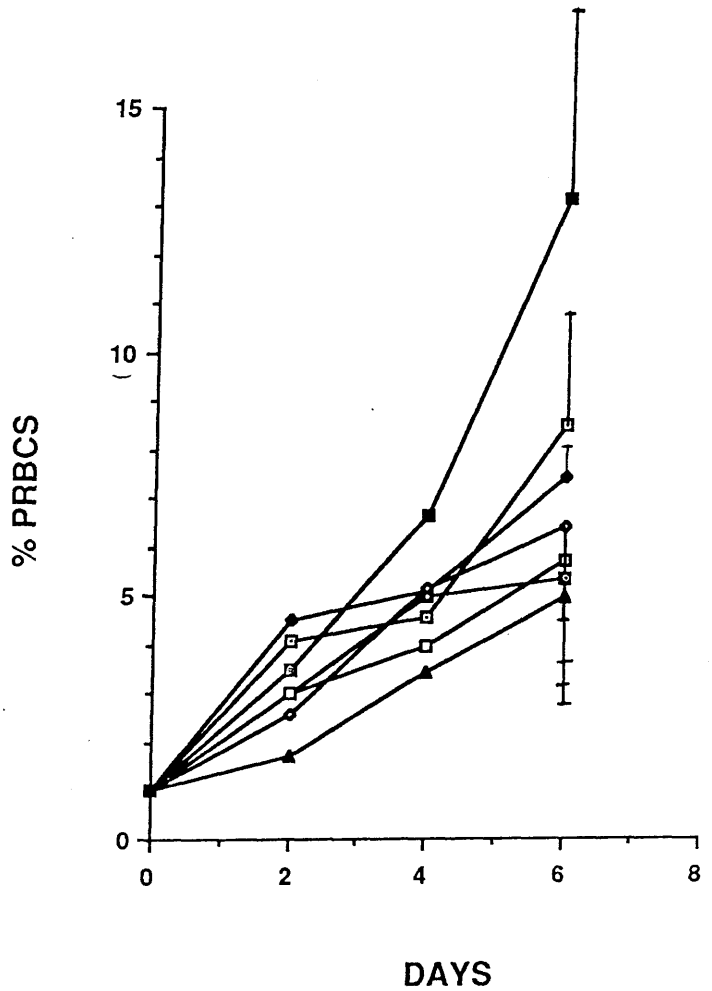
2 mg/l L-cysteine appears to be the optimal concentration supporting a mean parasitaemia of 4.5% in cultures of BG compared with 6.4% in cultures with PWCS. In cultures of RB medium containing 3 mg/l L-cysteine supported a mean parasitaemia of 13.3% which was higher than cultures with PWCS at 8.6%.

FIGURE 7.7 THE CULTIVATION OF ISOLATE BG IN MEDIUM SUPPLEMENTED WITH L-CYSTEINE AT 12 HOUR INTERVALS.



Isolate BG was grown in a 96 well microtitre plate at a 10% haematocrit in 200µl aliquots of complete medium supplemented with 24 ◆ , 12 ◘ , 6 ◆ , 3 ■ or 1.5 ◘ mg/l L cysteine. This medium was freshly prepared every 12 hours immediately before use. BG was also grown under the same conditions in normal medium both with ◘ and without ▲ $2x10^6$ PWCS/ml. Each treatment was carried out in triplicate. Bars indicate spread of points.

FIGURE 7.8 THE CULTIVATION OF ISOLATE RB IN MEDIUM SUPPLEMENTED WITH L-CYSTEINE AT 12 HOUR INTERVALS.



Isolate RB was grown in a 96 well microtitre plate at a 10% haematocrit in 200 µl aliquots of complete medium supplemented with 24 ◆ , 12 ◻ , 6 ○ , 3 ■ or 1.5 ◻ mg/l L cysteine. This medium was freshly prepared every 12 hours immediately before use.. RB was also grown under the same conditions in normal medium both with ◻ and without ▲ $2x10^6$ PWCS/ml. Each treatment was carried out in triplicate . Bars indicate spread of points.

Growth of P. falciparum with 2-Mercaptoethanol Supplemented Medium

Experiment 7.3A

Isolate AF was grown at a 10% haematocrit in wells containing 200 µl of complete medium supplemented with 5×10^{-4}, 1×10^{-4}, 5×10^{-5}, 1×10^{-5}, 5×10^{-6} or 1×10^{-6} molar 2-mercaptoethanol (2-ME) in a 96 well microtitre plate. AF was also grown under the same conditions in normal complete medium and also in medium containing 2×10^{6} PWCS/ml. Each treatment was carried out in triplicate and the starting parasitaemia of AF was 0.5%.

The 2-ME supplemented medium was prepared on day 0 and was used throughout the experiment. 150 µl of medium from each well was replaced daily and smears were made on days 2, 3, 6 and 7.

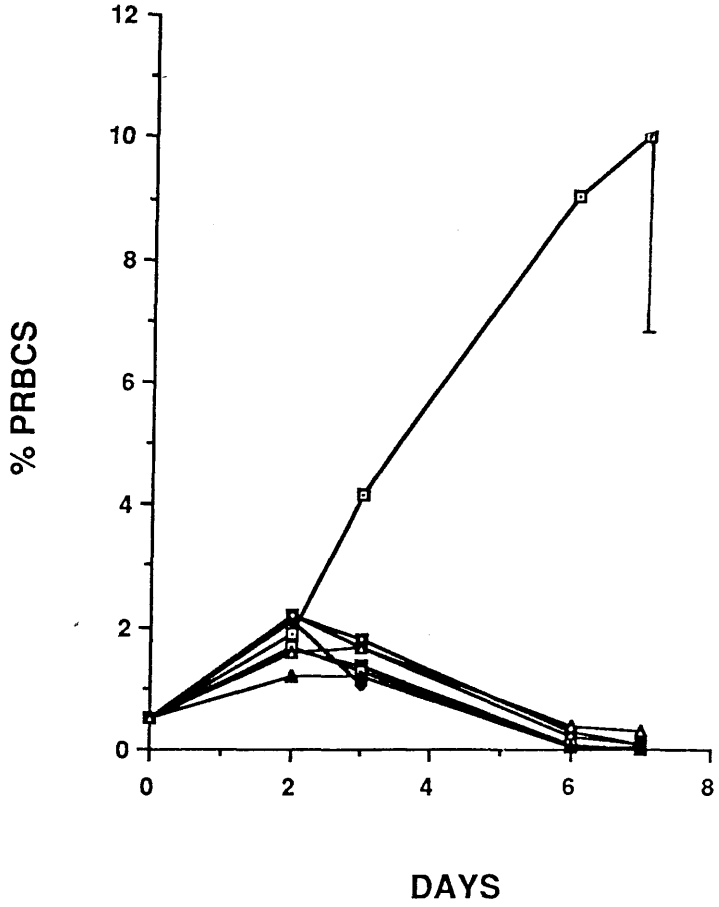
The experiment was repeated using isolate AF with a starting parasitaemia of 0.5%.

Results

The 2-ME supplemented medium did not promote a sustained increase in the growth of isolate AF (Figures 7.9 and 7.10). Cultures grown in medium containing 1×10^{-4} and 5×10^{-5} molar 2-ME (see Figure 7.9) supported mean parasitaemias of 0.1% on day 7, while cultures with 1×10^{-6} molar 2-ME supported a parasitaemia of 0.03%, compared with 10.1% in cultures with PWCS and 0.3% in control cultures (normal medium no PWCS).

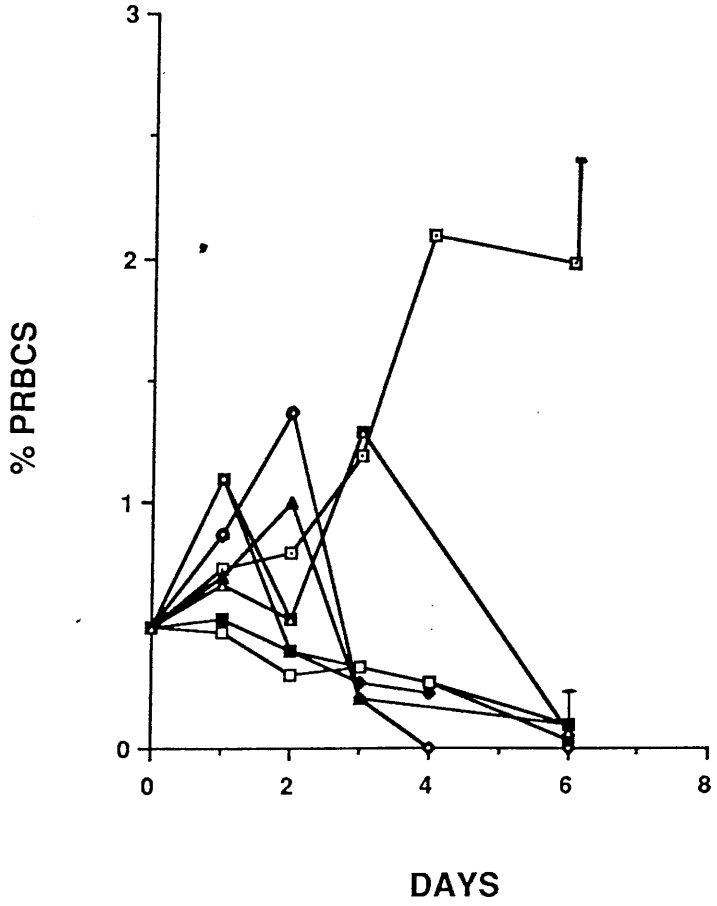
A similar pattern was observed when the experiment was repeated using isolate AF (Figure 7.10)

FIGURE 7.9 THE CULTIVATION OF ISOLATE AF IN MEDIUM SUPPLEMENTED WITH 2 MERCAPTOETHANOL.



Isolate AF was grown in a 96 well microtitre plate at a 10% haematocrit in 200 µl aliquots of complete medium supplemented with $5x10^{-4}$ ◆ $1x10^{-4}$ ■ $5x10^{-5}$ ◇ $1x10^{-5}$ ■ $5x10^{-6}$ □ or $1x10^{-6}$ ▲ molar 2- mercaptoethanol. This medium was prepared on day 0 and used throughout the experiment. AF was also grown under the same conditions in normal medium both with □ and without ▲ $2x10^{6}$ PWCS/ml. Each treatment was carried out in triplicate . Bars indicate spread of points.

FIGURE 7.10 THE CULTIVATION OF ISOLATE AF IN MEDIUM SUPPLEMENTED WITH 2 MERCAPTOETHANOL.



Isolate AF was grown in a 96 well microtitre plate at a 10% haematocrit in 200 µl aliquots of complete medium supplemented with $1x10^{-4}$ $5x10^{-5}$ $1x10^{-5}$ $5x10^{-6}$ $1x10^{-6}$ or $x10^{-7}$ molar 2- mercaptoethanol. This medium was prepared on day 0 and used throughout the experiment. AF was also grown under the same conditions in normal medium both with and without $2x10^{6}$ PWCS/ml. Each treatment was carried out in triplicate. Bars indicate spread of points.

Experiment 7.3B

In this experiment the 2-ME supplemented medium was freshly prepared every 24 hours immediately before use. Isolate RC was grown as described above with a starting parasitaemia of 0.5%. 150 µl of medium from each well were replaced every 24 hours.

Results

All the 2-ME supplemented media supported some parasite growth on day 6 (see Figure 7.11). Medium containing 1 x 10^{-5} molar 2-ME supported the most parasite growth with a mean parasitaemia of 1.5% on day 6 followed by 1 x 10^{-6} molar and 5 x 10^{-6} molar medium, both with mean parasitaemias of 0.7%. These were higher than control cultures (normal medium, no PWCS) at 0.6%. Medium supplemented with 5 x 10^{-4}, 1 x 10^{-4} and 5 x 10^{-5} molar 2-ME all supported less parasite growth than control cultures on day 6.

Experiment 7.3C

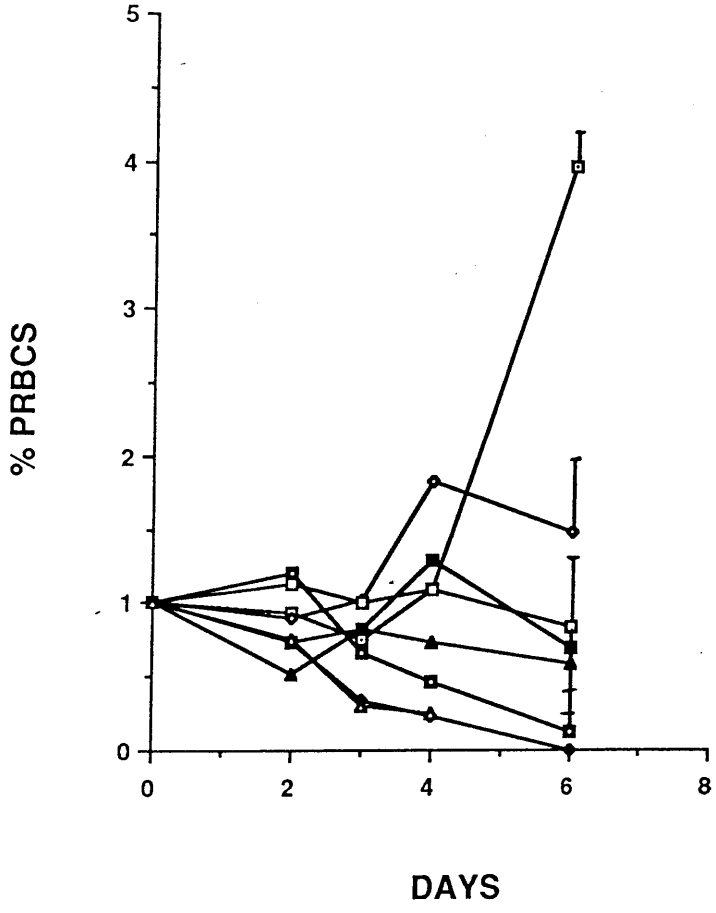
In this experiment the 2-ME supplemented medium was freshly prepared every 12 hours immediately before use.

Isolate API was grown as described above with a starting parasitaemia of 0.4% and 150 µl of medium from each well were replaced every 12 hours.

Result

Cultures of API with 2-ME supplemented medium, did, with the exception of 1 x 10^{-4} molar medium, achieve higher parasitaemias than

FIGURE 7.11 THE CULTIVATION OF ISOLATE RC IN MEDIUM SUPPLEMENTED WITH 2 MERCAPTOETHANOLAT 24 HOUR INTERVALS.



Isolate RC was grown in a 96 well microtitre plate at a 10% haematocrit in 200µl aliquots of complete medium supplemented with $5x10^{-4}$ ◆ $1x10^{-4}$ ▫ $5x10^{-5}$ ◇ $1x10^{-5}$ ■ $5x10^{-6}$ □ or $1x10^{-6}$ ▲ molar 2- mercaptoethanol. This medium was prepared every 24 hours immediately before use. RC was also grown under the same conditions in normal medium both with ▫ and without △ $2x10^6$ PWCS/ml. Each treatment was carried out in triplicate . Bars indicate spread of points.

control cultures on day 5 (see Figure 7.12). Medium containing 5 x 10^{-5} and 1 x 10^{-5} molar 2-ME supported a parasitaemia of 0.83%, but this was still lower than cultures containing PWCS with a mean parasitaemia of 1.4% on day 5.

Discussion

PWC conditioned medium which had been frozen and stored at -20°C before use was not effective in promoting an increase in the multiplication rate of *P. falciparum*. Medium which had been in contact with PWCS for 24 hours immediately before use was however able to stimulate some increase in parasite multiplication, although this was less than that which was promoted by PWCS.

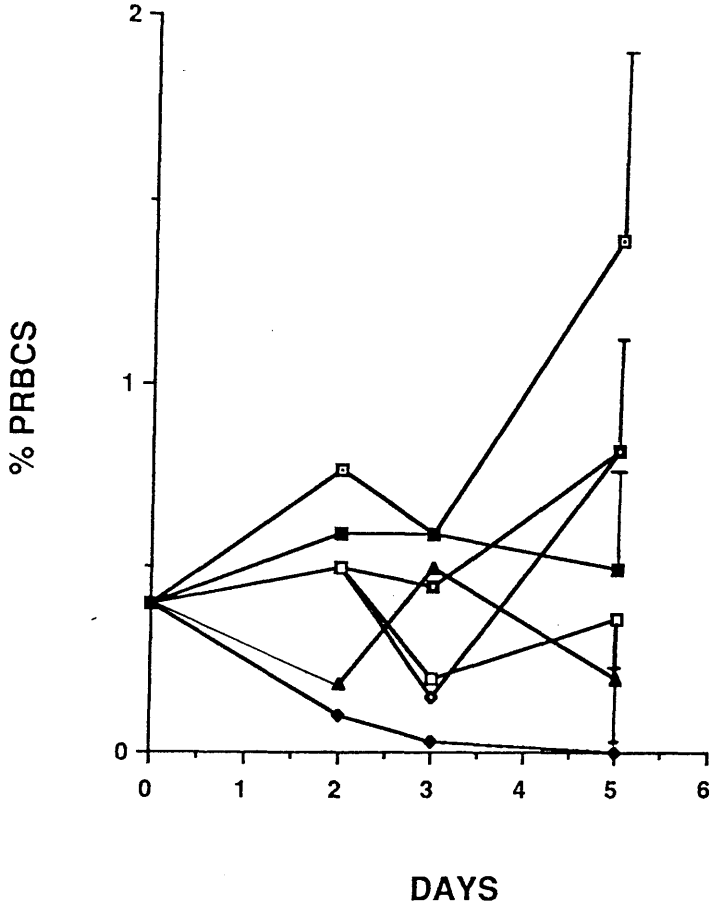
Tanner (1980) also found that mammalian fibroblast conditioned medium would not support the growth of bloodstream form trypanosomes.

These findings do, however, contrast with those of Mazier *et al.* (1984) who observed that medium conditioned by rodent hepatocytes gave *P. falciparum* multiplication rates only slightly inferior to those in cultures with rodent hepatocytes as feeder cells even when the medium had been frozen before use. This suggests that different mechanisms are involved in promoting increased parasite growth in each system.

Ishii *et al.* (1981) report that mouse lymphoma cells (L1210) will grow in the presence of a feeder cell layer of IMR90 cells. Without IMR90 cells the L1210 cells do not grow and their viability decreases rapidly. These workers have also shown that culture medium which had been in contact with IMR90 cells for

FIGURE 7.12 THE CULTIVATION OF ISOLATE AP1 IN MEDIUM SUPPLEMENTED WITH 2 MERCAPTOETHANOL AT 12 HOUR INTERVALS.

FIGURE 7.12



Isolate AP1 was grown in a 96 well microtitre plate at a 10% haematocrit in 200 µl aliquots of complete medium supplemented with $1x10^{-4}$ ◆ $5x10^{-5}$ ■ $1x10^{-5}$ ◇ $5x10^{-6}$ ▲ or $1x10^{-6}$ □ molar 2- mercaptoethanol. This medium was prepared every 12 hours immediately before use. AP1 was also grown under the same conditions in normal medium both with ■ and without ▲ $2x10^{6}$ PWCS/ml. Each treatment was carried out in triplicate. Bars indicate spread of points.

between 3 and 24 hours under normal culture conditions had a slight but significant effect on promoting the growth and viability of L1210 cells. This effect was improved by increasing the time of conditioning. However when the medium was stored at $4^{o}C$ for 1 day it no longer had a stimulatory effect on the L1210 cells. These findings are similar to those observed with PWC conditioned medium and cultures of P. falciparum.

Ishii et al. (1981) suggest that IMR 90 cells are producing a growth and/or viability factor and that this factor is unstable, this would also appear to be the case with PWCS.

L1210 cells were not easily adapted to culture, but they were successfully established in vitro without feeder cells using medium supplemented with 1mM L-cysteine and 0.3mM 1/2 L-cysteine [Dixon et al. 1966]. Broome and Jeng (1972) also cultured L1210 cells in the medium of Balk (1971) and discovered that the growth promoting component of the medium was cysteine.

Duszenko et al. (1985) have also found that the requirement of bloodstream form trypanosomes for feeder cells can be replaced by medium supplemented with L-cysteine. When cultures of P. falciparum were grown in medium supplemented with L-cysteine at 12 hour intervals an increase in parasite growth was observed. The concentration of L-cysteine which promoted optimal parasite multiplication was 2.0×10^{-5} molar which is close to the cysteine concentration in human serum at approximately 1.3×10^{-5} [Brigham et al. 1960]. This evidence suggests that one role of PWCS as feeder cell is to supply L-cysteine. This hypothesis is supported by the observation that the growth of L1210 cells can be promoted

mouse peritoneal macrophages [Nathan and Terry 1975, Ishii et. al. 1981] indicating that these feeder cells can supply the L-cysteine required by the L1210 cells. Further evidence is provided by Ishii et al. (1981) who have also shown that mouse peritoneal macrophages produce thiols in vitro.

Under standard cultures cysteine is rapidly oxidised to cyst ine. Toohey (1975) has shown that 2.5×10^{-3} molar cysteine in MEM is oxidised to cystine within 4 hours.

Duszenko et al. (1985) have shown that trypanosomes can only utilize cysteine and not cystine. Ishii et al. (1981) found that when cystine was added to culture medium under the same conditions as cysteine no growth promoting effect on L1210 cells was observed.

It would appear that cysteine is able to promote an increase in cell growth but is no longer able to do so when oxidised to cystine.

This would explain why conditioned medium which had been stored before use was not able to promote increased parasite growth and why cysteine supplemented medium was only effective when freshly prepared every 12 hours.

Duszenko et al. (1985) found that the addition of 2-mercaptoethanol (2ME) to culture medium would allow the growth of trypanosomes while Broome & Jeng (1973) and Ishii et al. (1981) report that L1210 cells will also grow without feeder cells in medium containing 2ME.

Nathan & Terry (1975) showed that the DNA synthesis of a number of murine lymphomas was stimulated in vitro by normal mouse peritoneal macrophages and that these lymphomas could also be stimulated by 2ME.

When 2ME was added to culture medium, it was found to be less effective than cysteine or PWC's in promoting the growth of P. falciparum.

When medium containing 2-ME was prepared on day 0 and used throughout the experiment the growth of P. falciparum over the first 2-3 days was greater than in control cultures, however, parasite growth declined rapidly after 2-3 days. This decline in parasite growth did not occur when medium containing 2ME was prepared immediately before use. The effect of 2ME supplemented medium was not increased when the medium was freshly prepared every 12 hours rather than every 24 hours as was the case with cysteine supplemented medium. This may be due to the fact that cysteine has a half life of only 2 hours unlike that of 2ME which is 40 hours.

Duszenko et al. (1985) suggest that 2ME sustains the growth of trypanosomes by reducing cystine to cysteine.

Ohmori & Yamamato (1982) have reported that cystine was incorporated into murine lymphocytes 5 to 6 times more slowly than cysteine but that the rate of cystine uptake was accelerated in the presence of 2ME. Ishii et al. (1981) have suggested that one role of 2-ME is to facilitate the uptake of cystine.

2ME was found to be less effective in promoting the growth of P. falciparum than of other cell types. It may be that the concentration of cystine in the RPMI (0.19mM) is not the optimum required for the reaction with the amount of 2ME added to the medium.

It has been shown that L1210 cells [Ishii et al. 1981] and trypanosomes [Duszanko et al. 1985] can be cultivated in normal medium in the presence of feeder cells or without feeder cells in medium supplemented with cysteine, suggesting that the feeder cells may be providing cysteine in vitro.

Mouse peritoneal macrophages have been shown to act as feeder cells for L1210 cells [Nathon & Terry 1975, Ishii et al. 1981] and for cultures of P. falciparum, and in both of these systems can be replaced by the addition of cysteine supplemented medium at 12 hour intervals. The evidence indicates that cysteine is at least one of the factors provided by PWCS which is responsible for promoting an increase in parasite growth.

CHAPTER 8

Discussion

Discussion

A major advancement in the field of malaria research has been the development of the technique by Trager & Jensen (1976) for the long term *in vitro* cultivation of the asexual erythrocytic stages of *P. falciparum*. Most isolates, however, grow poorly during the first weeks of cultivation and only some can be successfully established in culture [Chin & Collins 1980, Trager & Jensen 1976].

It is now well documented that natural erythrocytic infections of *P. falciparum* are mixtures of genetically diverse parasites [Reviewed by Walliker 1983]. It would seem likely that when an isolate is slow to adapt to culture, a certain amount of selection is taking place and only a proportion of the original parasite population is able to make the transition to continuous growth *in vitro*. There is considerable evidence to support this, a number of workers having shown that the parasite population does undergo certain changes during continuous culture. Rosario (1981) has reported changes in the enzyme patterns of an isolate within the first 2 weeks of *in vitro* cultivation. Reports of changes in the susceptibility of cultured parasites to antimalarials and the ability of parasites to develop into gametocytes have also been made [Jensen *et al.* 1981, Ponnudurai *et al.* 1982]. Langreth *et al.* (1979) observed the loss of knob structures from infected erythrocytes during *in vitro* cultivation. This has been shown to correlate with the loss of the property of cytoadherence of PRBCS to human endothelial cells [Udeinya *et al.* 1983], or amelanotic melanoma cells [Schmidt *et al.* 1982].

The loss of the property of cytoadherence is important as it

implies that the parasites studied and cultured *in vitro* are those most able to adapt to life *in vitro* under standard culture conditions, rather than those which may predominate in the natural host. The conservation of the entire parasite population is an important aim if *P. falciparum* cultures are to be used for biochemical analysis, for drug screening or for antigen production.

It has been demonstrated here that the presence of a feeder cell layer of mouse PWCS promotes increased parasite multiplication rates in new and established isolates of *P. falciparum*. The increase in parasite multiplication rate is due to an increase in the number of PRBCS, rather than due to the destruction of non-infected RBCS by PWCS, or through the spontaneous lysis of RBCS in cultures which could have given a false impression of an increasing parasitaemia.

The effect of PWCS on parasite proliferation was most marked with new isolates. The use of feeder cells allows some new isolates of *P. falciparum* to be established more readily *in vitro*. Of 13 isolates tested, only 4 could not be established in culture with PWCS, and 3 of these isolates were taken from patients who had been treated with antimalarials prior to blood collection and so the parasites may have already been killed by the drug.

These findings agree with those of Mazier *et al.* (1984) who report that out of 9 new isolates tested, only 3 could be established under standard culture conditions whereas all 9 isolates grew readily in the presence of a feeder cell layer of

rodent hepatocytes. Phillips et al. (1987) also report that new isolates of P. falciparum adapt to culture more readily in the presence of a feeder cell layer of human peripheral mononuclear cells (PBMNS).

The faster adaptation of P. falciparum to culture in the presence of feeder cells may be due to an increased number of individual parasites adapting to culture conditions (suggested by Mazier et al. 1984). Alternatively the presence of a feeder cell layer may increase the multiplication rate of the parasites which would normally have adapted anyway. To explore the first possibility isolate RC was examined before culture and after 141 days in culture with or without PWCS, using a bank of monoclonal antibodies (McAbs) to P. falciparum in an IFAT test.

The McAbs were prepared by McBride et al. (1982) against 2 P. falciparum isolates from Thailand (K1 and PB1) and they have subsequently been used to demonstrate considerable antigenic diversity between and within individual isolates of P. falciparum [McBride et al. 1982, 1985].

The immunofluorescence test was carried out when RC was first isolated from a patient. Parasites in the original isolate reacted with 2 McAbs which recognise schizont antigens of 200 kDa (13.1 and 12.14) and also with a McAb (9.21) which recognises a 90 kDa parasite antigen. After 141 days in culture with PWCS the parasites still reacted with McAbs 13.1 and 12.1, but not with 9.21. The parasites which had been cultured without PWCS did not react with any of these McAbs, indicating that the parasite

populations expressing the antigens recognised by these McAbs had either been lost or had decreased in number so that they were no longer detectable in the IFAT test. In continuing studies it would be of interest to examine antigen slides which were prepared from cultures of isolate RC after it had been maintained in culture for various periods of time to determine approximately when the parasites expressing these antigens were lost.

The evidence presented here supports the option that PWCS allow the survival of more parasite types from the original isolate leading to increased diversity within cultures. Although more parasites may survive the initial isolation process in the presence of PWCS, it may be, however, that faster growing lines may then out grow lines which grow more slowly *in vitro*. Taking this last point into consideration an attempt was made to clone parasites directly from an infected blood sample, or after a short cultivation period, using a feeder cell layer of PWCS.

The results obtained when new isolates were cloned in the presence of a feeder cell layer of PWCS were variable, but when cloning was achieved the presence of PWCS appeared to greatly increase cloning efficiency. In experiments where no clones were produced it was later found that either the brand of RPMI used gave suboptimal parasite growth or, that the isolate as a whole did not adapt to culture.

Isolate AF was successfully cloned, in the presence of a feeder cell layer of PWCS, from a cryopreserved blood sample. A total of 16 clones were obtained and the antigenic diversity of 14

of these was investigated using McBride's McAbs as described above. These McAbs indicated that the original isolate was a mixture of different antigenic types and 3 different antigenic types could be recognised in the different clones. Three McAbs recognising a schizont antigen of 200 kDa gave positive results with the original isolate but did not react with any of the cloned parasite populations. This suggests that the parasite populations expressing these antigens, which are present in the original isolate, were not cloned. This could simply be due to chance, or because the parasites still have not adapted for some reason or even because the relevant antigens have undergone variation.

Three McAbs did not appear to react with parasites in the original isolate, but did give positive results with some of the cloned parasites. Parasites expressing the relevant antigens may have been present in the original isolate at very low frequencies and so could not be detected using a standard IFAT test.

A number of these McAbs have been used by McBride et. al. (1985) (along with other McAbs) to identify a family of proteins of between 190 and 200 kDa which are structurally and antigenically diverse and which have been shown to be responsible for much antigenic diversity in p. falciparum.

The culture supernatants of isolate AF and its associated clones were also investigated with regard to their S antigens. S antigens are soluble malarial proteins which are released into the plasma or culture medium when erythrocytes rupture to release merozoites and which retain their stability and immunoreactivity after treatment at 100°C [Wilson et al. 1969]. This work was

carried out by N. Bazarga at the Walter and Elisa Hall Memorial Institute of Medical Research, Melbourne, Australia. It was found that the culture supernatants from the majority of the parasite clones (15/16) and from the original isolate contained the same S antigen of 145-155 kDa, while in 1 cloned line this was approximately 10 kDa larger [N. Bazarga, personal communication]. These cloned parasites, therefore, show less antigenic diversity with regard to their S antigens than is demonstrated by the McAbs against blood stage antigens. Thaithong et. al. (1984) have also found a great deal of antigenic diversity by cloning an isolate of P. falciparum and testing with the same batch of McAbs [McBride et. al. 1982]. In 10 clones of 1 isolate from Thailand they recognised at least 5 different combinations of antigenic markers. These clones were also examined with regard to other characters, such as, isoenzymes, susceptibility to antimalarials, the presence or absence of knobs on infected erythrocytes, and proteins, and at least 7 different types of clone were found. This illustrates the large amount of phenotypic variation which can be seen within a single isolate. Thaithong et al. (1984) do not report how long this Thai isolate had been in culture before cloning was carried out, but it is likely that the period was greater than 24 hours which was the case with isolate AF.

It appears to be the adherent (macrophage enriched) PWC population which is most effective in promoting increased parasite growth. This is in agreement with the findings of Phillips et al. (1987) who successfully used a feeder cell layer of PBMNS for cultures of P. falciparum. Mouse PWCS have been used as feeder cells in other culture systems. Nathan & Terry (1975) used mouse

peritoneal macrophages as a feeder cell layer for cultures of mouse lymphoma L1210 cells. Butcher & Clancy (1984), however, found that human PBMNS could be stimulatory or inhibitory to cultures of P. falciparum. The factor which was inhibitory to parasite growth appeared to be released from monocytes within the first 24 hrs of their culture and affected intracellular parasite growth in a mode similar to that described by Jensen et al. (1983) for human Sudanese sera. Because of these similarities Butcher & Clancy (1984) suggest that the 2 molecules may be the same. It has been reported that a large number of factors are released by monocytes and macrophages, including tumour necrosis factor (TNF) [Clark 1978], polyamine oxidase [Morgan & Christenson 1983] and free radicals [Clark & Hunt 1983] and all of these factors may be cytotoxic to malaria parasites. Mathews (1981) has shown that factors cytotoxic to tumour cells are also released from human monocytes during the first 24 hours of their culture.

There is no evidence to suggest that a parasite inhibitory factor is released from peritoneal macrophages. It is interesting to note that when PWCS were cultured prior to use and the culture medium was not replenished until 24 hours after parasites were added to the PWCS, 3 day old PWCS supported slightly less parasite multiplication than 0, 1 or 5 day old PWCS. This suggests that a factor which li mits parasite multiplication may have been accumulating in the culture medium. PWCS cultured under the same conditions for 5 days before use supported higher parasitaemias than the 3 day old PWCS, which does not, however support this suggestion. One possible explanation of these results may be that the PWCS are utilizing some essential growth factor and once the

levels of this growth factor fall below a certain level, they produce this factor which is then released into the culture medium.

It has been shown that mouse PWCS can be used successfully as feeder cells in cultures of P. falciparum. It would, nevertheless, be more convenient if a suitable cell line could be found to replace PWCS and dispense with the need to use mice as a source of feeder cells. Three human cell lines were, therefore, tested as potential feeder cells for cultures of P. falciparum, and were selected on account of their availability and ease of cultivation; they were Raji, 143 and K562. In the system used these cell lines were found not to be effective as feeder cells.

Mazier et al. (1984) also reported testing a number of cell lines as potential feeder cells for cultures of P. falciparum. This group tested 2 human hepatoma cell lines (HEPG2 and PLC PRFS), 1 rat hepatoma cell line (FAZA), human embryonic and liver fibroblasts and rat liver epithelial cells, but in the system used these cells were not effective in promoting an increase in parasite growth. This group did, however, successfully use a feeder cell layer of rodent hepatocytes co-cultivated with a liver epithelial cell line to promote increased growth of P. falciparum. Mazier et al. 1984 suggest that hepatocytes may act as feeder cells by removing toxic metabolites from the culture medium or by supplying nutrients which are either absent from the culture medium or are only present in a small amounts. Hepatocytes have the capacity to produce a number of factors including proteins and they can also recycle lactic acid via the Krebs cycle. This was

considered important as malaria parasites produce lactic acid [Jensen et al. 1983B] which causes acidification of the culture medium and this can lead to an inhibition of parasite growth. This initial hypothesis is not, however, consistent with the results obtained with hepatocyle culture supernatants. Hepatocyte conditioned medium promoted parasite multiplication rates only slightly inferior to those promoted by cultures with hepatocytes as feeder cells. Mazier et al. (1984) also report that, from these results, it appears that in this system the observed improvement in parasite multiplication is related to a function of fully differentiated hepatocytes as, no feeder cell effect was seen with fibroblasts or epithelial cells. Many of the functions exhibited by hepatocytes are also shown by the highly differentiated human liver carcinoma HEP G2. However Mazier et al. (1984) found that this cell line leads to accelerated medium exhaustion because it continues to grow rapidly once the cells have reached confluence. This may have also been a problem with the Raji, 143 and K562 cells when they were tested as feeder cells.

Although human fibroblasts were not effective as feeder cells for cultures of P. falciparum [Mazier et al. 1984], fibroblasts have been used as feeder cells in a variety of other systems. Human fibroblasts have successfully been used as feeder cells for cultures of rodent parenchymal hepatocytes [Michalopoulos et al. 1979] and mouse lymphoma cells [Rheinwold and Green 1975]. Mammalian fibroblasts have also been used as feeder cells for the successful cultivation of bloodstream form trypanosomes [Hirumi et al. 1977, Brun et al. 1981]. Brun et al. (1981) used

fibroblast like cells derived from the embryos of New Zealand white rabbits or from the mountain vole, Microtus monitanus. M. montanus cells were tested as feeder cells for cultures of P. falciparum and were found to be as effective as PWCS in promoting an increase in parasite multiplication.

Efforts have been made to determine the mode of action of feeder cells on cultures of bloodstream stage trypanosomes. Tanner (1980) found that fibroblast conditioned medium, which was not frozen before use, was not effective in promoting sustained trypanosome growth. Parasites cultured in 3 hr conditioned medium died within 6 hrs, 6 hr conditioned medium had a slight effect on parasite survival and 12 hr conditioned medium supported the survival of trypanosomes as infectious bloodstream forms for up to 48 h. But this was at a much lower level than that of trypanosomes cultured with a feeder cell layer. PWC conditioned medium which had been stored at -20^{o}C before use was also found to be ineffective in promoting an increase in the multiplication rate of P. falciparum. These findings contrast with those of Mazier et al. (1984) who found that medium conditioned by hepatocytes gave parasite multiplication rates only slightly inferior to those in cultures with hepatocytes as feeder cells, even when the medium had been stored at -20^{o}C before use.

It is interesting to note that medium which had been in contact with PWCS for 24 hrs immediately before use was able to stimulate some increase in the multiplication rate of P. falciparum, although this was less than that promoted by PWCS. Ishii et al. (1981) have also shown that culture medium which had

been in contact with feeder cells, (IMR 90 cells) for between 3 and 24 hours under normal culture conditions had a slight but significant effect on promoting the growth and viability of L1210 cells. This effect was improved by increasing the time of conditioning. When the medium was stored at $4^{o}C$ for 24 hrs before use it, no longer, however, had a stimulatory effect on the L1210 cells.

It appears therefore that conditioned medium has similar properties in all these systems. Both Ishii et al. (1981) and Tanner (1980) suggest that their feeder cells are producing a growth and/or viability factor and that this factor is unstable. This would also appear to be the case with PWCS.

When parasitised erythrocytes were separated from the feeder cell layer of PWCS by a Millipore filter, parasite multiplication was inhibited. A similar result has been found by Tanner (1980) in the fibroblast-trypanosome culture system, who showed that the inhibition of growth was not due to adverse reactions to the material of the filters. In the P. falciparum PWC system the inserts alone were found to be inhibitory to parasite multiplication; it was therefore impossible to confirm Tanners conclusion that the decreased parasite multiplication was due to too great a diffusion path between the two cell types.

The requirement of bloodstream form trypanosomes for feeder cells in vitro can be replaced by medium supplemented with L cysteine [Duszenko et al. 1985]. Mouse lymphoma L1210 cells have also been successfully established in culture without feeder cells in medium supplemented with L cysteine [Dixon et al. 1966, Broome

& Jeng 1972]. When cultures of P. falciparum ere grown, without PWCS, but in medium which was supplemented with L cysteine at 12 hour intervals an increase in parasite g rowth was observed. It therefore appears that one role of PWCS as feeder cells may be to supply L cysteine continuously. Support for this hypothesis is provided by the observation that the growth of L1210 cells can be promoted by mouse peritoneal macrophages [Nathan & Terry 1975, Ishii et al. 1981] indicating that these feeder cells can supply the cysteine required by the L1210 cells. Further evidence is provided by Ishii et al. 1981 who have also shown that mouse eritoneal macrophages produce thiols in vitro.

It is worth noting at this point that irradiated PWCS were found to be less effective than normal PWCS in promoting an increase in parasite multiplication. Irradiation causes the production of ionized molecules from biomolecules, so it leads to the formation of extremely unstable ions or free radicals when it passes through living matter [Bridges 1976] and it is also known that irradiation can cause various kinds of structural alterations and lesions in cellular DNA [Lehninger 1982]. It is likely therefore, that irradiated peritoneal macrophages had been disrupted metabolically and this may have impaired their ability to produce thiols in vitro.

Cysteine is an essential amino acid for many types of cultured mammalian cells and most culture media contain cysteine or cystine. Cysteine is however unstable and is rapidly converted to cystine by auto-oxidation [Toohey 1975].

Duszenko et al. (1985) have shown that trypanosomes can only utilize cysteine and not cystine, and Ishii et al. (1981) found

that when cystine was added to culture medium under the same conditions as cysteine no growth promoting effect on L1210 cells was observed. This may explain why PWC conditioned medium which had been stored before use was not able to promote increased parasite growth and why cysteine supplemented medium was only effective when freshly prepared every 12 hrs. In both cases the cysteine may have been oxidised to cystine.

Malaria parasites can only synthesise a limited number of amino acids, but both parasites and infected erythrocytes have the ability to take up free amino acide from the external environment. Cysteine is present in human serum at low levels but even when supplied at higher levels is poorly incorporated into parasite proteins [Sherman 1979]. It is interesting to note at this point that the concentration of L cysteine which promoted the greatest increase in parasite multiplication was 2.0×10^{-5}m which is close to the cysteine concentration in human serum at approximately 1.3×10^{-5}m [Brigham *et al*. 1960].

It has been shown that *P. knowlesi* infected erythrocytes accumulate cystine, along with isoleucine, methionine, leucine and histidine, (Sherman 1979) but no data regarding the uptake of cystine and cysteine by *P. falciparum* infected erythrocytes has been published.

Duszenko *et al*. 1985 found that the addition of reducing agents such as 2-mercaptoethanol (2ME) or thioglycerol to culture medium would support the growth of trypanosomes, while Broome & Jeng (1973) and Ishii *et al*. (1981) report that L1210 cells will also grow without feeder cells in medium containing 2ME.

Nathan and Terry (1975) have shown that the DNA synthesis of a number of murine lymphomas was stimulated *in vitro* by normal mouse peritoneal macrophages, and that these lymphomas could also be stimulated by 2ME. Baltz *et al.* (1985) also reported the growth of *T. brucei*, *T. equiperdum*, *T. rhodesiense* and *T. gambiense* axenically in media supplemented with 2ME. However, in this study 2ME supplemented medium was found to be less effective than either cysteine or PWCS in promoting increased multiplication of *P. falciparum*.

Dusenko *et al.* (1975) suggest that 2ME sustains the growth of trypanosomes by reducing cystine (which cannot be utilized by trypanosomes) to cysteine. The results of Ishii *et. al.* (1981) suggest that the most important role of 2ME is to enhance the utilization of extracellular cystine by the cells. But the function of 2 ME does not appear to be merely the production of cysteine from cystine in the medium. These workers also suggest that a mixed disulphide of 2ME and cysteine is formed in the culture medium and is taken up by the L1210 cells at a relatively high rate. The 2ME may be used repeatedly as a carrier of cysteine. This latter suggestion is of particular interest as Sherman and Tanigoshi (1974) have shown that cysteine enters *P. lophurae* parasites by what appeared to be a carrier mediated process. It may be that the concentration of cystine in the RPMI (0.19 mM) which was used for the cultivation of *P. falciparum* is not the optimum required for the reaction with the amount of 2ME which was added to the medium.

Preliminary experiments using cryopreserved PWCS were carried out with a view streamlining the use of PWCS when cloning new

isolates of P. falciparum as the preparation of large numbers of PWCS is time consuming and it is desirable to clone new isolates as quickly as possible. These preliminary experiments were disappointing however, as the ability of cryopreserved PWCS to promote an increase in the multiplication rate of P. falciparum cultures declined quickly, probably due to a decline in macrophage viability during storage at -70°C.

It has been shown that M. montanus cells can be as effective as PWCS in promoting increased multiplication in P. falciparum cultures. Therefore M. montanus cells may lead to an increase in the cloning efficiency of P. falciparum to the same extent as that observed with PWCS. It would be worthwhile carrying out cloning experiments using a feeder cell layer of M. montanus cells. These cells can be routinely maintained in culture and could be conveniently available at all times, so making the cloning technique simpler and less time consuming.

There are a number of other areas of work covered by this project which are worthy of further investigation.

It has been shown that the addition of cysteine to culture medium can promote an increase in parasite multiplication. It would, however, not be practical to use medium supplemented with cysteine for the routine maintenance of parasite cultures as the medium needs to be freshly prepared and replaced at intervals of not more than 12 hours to achieve an increase in parasite multiplication. But it may be that by adjusting the levels of cystine, cysteine and 2ME in RPMI that this medium could be improved for the cultivation of P. falciparum. As very little

data appears to be available on this subject it would be of interest to carry out uptake experiments using ^{35}S-labelled cystine and cysteine to determine if and at what rate these amino acids are taken up by P. falciparum infected erythrocytes.

The clones produced from isolate AF have only been characterised using a panel of McAbs (as described by McBride et. al. 1982) and with regard to their s-antigens. In view of the considerable diversity shown by the clones produced from a single Thai isolate [Thaithong et al. 1984] it would be worthwhile examining the clones produced from isolate AF with regard to other characters. These could include, isoenzymes, susceptibility to antimalarials, the presence or absence of knobs on the surface of infected erythrocytes, the ability to produce gametocytes and the pattern of proteins exhibited on 2-dimensional SDS PAGE gels.

Finally it would also be of interest to test cell lines which have been successfully used in other systems as feeder cells for P. falciparum cultures, particularly those cell lines used by Hirumi et al. (1979) for the cultivation blood stream from trypanosomes and the cell line IMR 90 which was used as a feeder cell by Ishii et al. (1981) for mouse lymphoma L1210 cells.

Appendix

Phosphate Buffers (PBS)

1. Isotonic PBS

Stock solution

60 g $Na_2HPO_4.12H_2O$

13.6 g $NaH_2PO_4.2H_2O$

8.5 g NaCl.

made up to 1 litre with distilled H_2O

0.9% Saline

9 g NaCl

made up to 1 litre with distilled H_2O

Buffer

40 mls stock

made up to 1 litre with 0.9% saline and adjusted to pH 7.2.

2. Giemsas' Buffer

3 g $Na_2 HPO_4$

0.6 g $KH_2 PO_4$

made up to 1 litre with distilled H_2O. pH adjusted to 7.2 with IN NaOH.

Media

1. RPMI

Stock

10.4 g RPMI 1640 powdered medium (with L-glutamine) (GIBCO).

5.94 g N_2-hydroxyethylpiperazine-N'-2 ethame sulphonic acid (Hepes) (SIGMA).

Made up to 960 mls with distilled H_2O and filter sterilized (Millipore filter size 0.22 um) and aliquoted into 100 ml bottles.

Incomplete RPMI

100 mls RPMI

4.2 mls 5% (w/v) $NaHCO_3$ (filler sterilized)

2.5 mg gentamycin sulphate (SIGMA)

Complete RPMI

90 mls Incomplete RPMI

10 mls Human AB serum

2. Minimal Essential Medium (MEM)

Stock

9.7 g MEM powdered medium (with non-essential amino acids, Earle's salts and L-glutamine) (SIGMA)

5.94 g Hepes (SIGMA)

2.2 g Na HCO_3

Made up to 1 litre with distilled H_2O (pH 7.2), filter sterilized (Millipore filter size 0.22 um) and aliquoted into 100 ml bottles.

Complete MEM

85 mls stock MEM

15 mls Heat inactivated rabbit serum

Miscellaneous

Sorbitol-glycerol

380 g glycerol

39 g sorbitol (BDH)

6.3 g NaCl

Made up to 1 litre with distilled H_2O and filter sterilised.

5% D-Sorbitol.

5 g D-sorbitol (BDH)

Made up to 100 mls in PBS (pH 7.2) and filter sterilized.

3.5% Saline

3.5 g NaCl

Made up to 100 mls in distilled H_2O and filter sterilized.

4.5% Saline

4.5 g NaCl

Made up to 100 mls in distilled H_2O and filter sterilized.

REFERENCES

References

Abele, D.C., Tobie, J.E., Hill, G.J., Contocos, P.G. and Evans, C.B. (1965). Alterations in serum proteins and 19S antibody production during the course of induced malarial infections in man. Am. J. Trop. Med. Hyg., 14, 191-197.

Adu, D., Williams, D.G., Quakyi, I.A., Voller, A., Anim-Addo, Y., Bruce-Tagoe, A.A., Johnson, G.D., and Holborrow, E.J. (1982). Anti ssDNA and anti-nuclear antibodies in human malaria. Clin. Expt. Immunol, 49, 310.

Aikawa, M., Miller, L.H., Johnson, J. and Rabbege, J. (1978). Erythrocyte entry by malarial parasites. A moving junction between erythrocyte and parasite. J. Cell. Biol. 77, 72-82.

Aikawa, M., Carter, R., Ito, Y. and Nijhout, M. (1984). New observations on gametogenesis, fertilization and zygote transformation in Plasmodium gallinaceum. J. Protozool. 31, 403-413.

Alger, N.E., (1968). In vitro development of Plasmodium berghei ookinetes. Nature, 218, 774.

Allison, A.C. and Eugui, E.M. (1982). A radical interpretation of immunity to malaria parasites Lancet, 2, 1431-1433.

Anders, R.F., Brown, G.V. and Edwards, A., (1983). Characterisation of an S-antigen synthesised by several isolates of P. falciparum. Proc. Natl. Acad. Sci. USA., 80, 6652-6656.

Balk, S.D., (1971). Stimulation of the proliferation of chicken fibroblasts by folic acid or a serum factor(s) in a plasma containing medium. Proc. Natl. Acad. Sci. USA., 68, 1689-1692.

Ballou, W.R., Hoffman, S.L., Sherwood, J.A., Hollingdale, M.R., Neva, F.A., Hockmeyer, W.T., Gordon, D.M., Schneider, I., Wirtz, R.A., Reeve, D., Diggs, C.L. and Chulay (1987). Safety and efficacy of a recombinant DNA P. falciparum sporozoite vaccine. Lancet, I. 1277-1281.

Ballou, W.R., Rothbard, J., Wirtz, R.A., Gordon, D.M., Williams, J.S., Gore, R.W., Schneider, I., Hollingdale, M.R., Beaudoin, R.L., Maloy, W.L., Miller, L.H. and Hockmeyer, W.T., (1985). Immunogenicity of synthetic peptides from circumsporozoite protein of P. falciparum. Science, 228, 996-999.

Baltz, T., Baltz, D., Giroud, C. and Crockett, J. (1985). Cultivation in a semi-defined medium of animal infective forms of Trypanosoma brucei, T. equiperdum, T. evansi, T. rhodesiense and T. gambiense. Eur. Mol. Biol. Org. Journal 4, 1273-1284.

Bannister, L.H., Butcher, G.A., Dennis, E.D. and Mitchell, G.H. (1975). Structure and invasive behaviour of P. knowlesi merozoites in vitro. Parasitology 71, 483-491.

Bannister, L.H., Butcher, G.A. and Mitchell, G.H. (1977). Recent advances in understanding the invasion of erythrocytes by merozoites of P. knowlesi. Bull. World Health Organ., 55, 163-169.

Barr, P.J., Gibson, H.L., Enea, V., Arnot, D.E., Hollingdale, M.R. and Nussenzweig, V. (1987). Expression in yeast of a P. vivax antigen of potential use in a human malaria vaccine. J. Expt. Med., 165, 1160-1171.

Bass, C.C. and Johns, F.M. (1912). The cultivation of malarial plasmodia (P. vivax and P. falciparum) in vitro. J. Expt. Med., 16, 567-569.

Beaudin, R.L. (1977). Should cultivated exoerythrocytic parasites be considered as a source of antigen for a malaria vaccine? Bull. World Health Organ., 55, 373-376.

Brasseur, P., Agrapart, M., Ballet, J.J., Druhile, P., Warrel, M.J. and Tharavanij, S., (1983). Impaired cell mediated immunity in P. falciparum infected patients with high parasitaemia and cerebral malaria. Clin. Immunol. Immunopathol., 27, 38-50.

Bray, R.S. and Garnham, P.C.C. (1982). The life cycle of primate malaria parasites. Br. Med. Bull., 38, 2, 117-122.

Bridges, B.A. (1976). Survival of bacteria following exposure to ultraviolet and ionizing radiations. Symp. Soc. Gen. Microbiol., 26, 183-208.

Briggs, N.T., Wellde, B.T. and Sadun, E.H. (1968). Variants of P. berghei resistant to passive transfer of immune serum. Expt. Parasitol., 22, 338-345.

Brigham, M.P., Stein, W.H. and Moore, S.(1960). The concentration of cysteine and cystine in human blood plasma. J. Clin. Invest., 39, 1633

Brockelman, C.R. (1982). Conditions favouring gametogenesis in the continuous culture of P. falciparum. J. Protozool., 29, 454-458.

Brockelman, C.R., Tan-Ariya, P. and Laovanitch, R. (1985). Observation on complete schizogony of P. vivax in vitro. J. Protozool., 32, 76-80.

Broome, J.D. and Jeng, M.W. (1972). Growth stimulation of mouse leukemia cells by thiols and disulfides in vitro. J. Natl. Cancer. Inst., 49, 579-581.

Brown, G.V., Culvenor, J.G., Crewther, P.E., Bianco, A.E., Coppel, R.L., Saint, R.B., Stahl, H.D., Kemp, D.J. and Anders, R.F., (1985). Localization of the ring infected erythrocyte surface antigen (RESA) of P. falciparum in merozoites and ring infected erythrocytes. J. Expt. Med., 162, 774-779.

Brown, I.N. (1969). Immunological aspects of malaria infection. Adv. Immunol., 11, 267-349.

Brown, I.N. and Phillips, R.S. (1974). Immunity to P. berghei in rats: Passive serum transfer and role of the spleen. Infect. Immun., 10, 1213-1218.

Brown, I.N., Allison, A.C. and Taylor, R.B. (1968). P. berghei infections in thymectomised rats. Nature, 219, 292-293.

Brown, J. and Smalley, M.E. (1980). Specific antibody dependent cellular cytotoxicity in human malaria. Clin. Expt. Immunol., 41, 423-429.

Brown, K.N. and Phillips, R.S. (1971). The role of thymus derived lymphocytes in protective immunity to malaria. Trans. R. Soc. Trop. Med. Hyg., 65, 7.

Brown, K.N., Berzins, K., Jarra, W. and Schetters, T. (1986). Immune responses to erythrocytic malaria. Clin. Immunol. Allergy, 6, 227-249.

Brun, R., Jenni, L., Schoenberger, M. and Schell, K.F. (1981). In vitro cultivation of bloodstream forms of T. brucei, T. rhodesiensie and T. gambiense. J. Protozool., 28, (4), 470-479.

Butcher, G.A. (1979). Factors affecting the in vitro culture of P. falciparum and P. knowlesi. Bull. World Health Organ., 57, (Suppl. 1), 17-26.

Butcher, G.A. and Clancy, R.L. (1984). Non-specific immunity to P. falciparum: in vitro studies. Trans. R. Soc. Trop. Med. Hyg., 78, 806-811.

Butcher, G.A. and Cohen, S. (1972). Antigenic variation and protective immunity in P. knowlesi malaria. Immunology, 23, 503-521.

Butcher, G.A., Mitchell, G.H. and Cohen, S. (1978). Antibody mediated mechanisms of immunity to malaria induced by vaccination with P. knowlesi merozoites. Immunology, 34, 77-86.

Capps, T.C. and Jensen, J.B. (1983). Storage requirements for erythrocytes used to culture P. falciparum. J. Parasitol., 69, 158-162.

Carter, R. and Miller, L.H. (1979). Evidence for environmental modulation of gametocytogenesis in P. falciparum in continuous culture. Bull. World Health Organ., 57, (Suppl. 1), 37-52.

Carter, R. and McGregor, I.A. (1973). Enzyme variations in P. falciparum in the Gambia. Trans. R. Soc. Trop. Med. Hyg., 67, 830-837.

Carter, R., Gwadz, R.W. and McAuliffe, F.M. (1979). P. gallinaceum, transmission blocking immunity in chickens II. The effect of antigamete antibodies in vitro and in vivo and their elaboration during infection. Expl. Parasitol., 47, 194-208.

Carter, E.H., Suhrbier, A., Beckers, P.J.A. and Sinden, R.E. (1987). The in vitro cultivation of P. falciparum ookinetes and their enrichment on Nycodenz density gradients. Parasitology, 95, 25-30.

Carter, R., Miller, L.H., Rener, J., Kaushal, D.C., Kumar, N., Graves, P.M., Grotendorst, C.A., Gwadz, R.W., French, C. and Wirth, D. (1984). Target antigens in malaria transmission blocking immunity. Phil. Trans. Roy. Soc. Lond., 307, 201-213.

Celada, A., Cruchaud, A. and Perrin, L.H. (1982). Opsonic activity of human immune serum on in vitro phagocytosis of P. falciparum infected red blood cells by monocytes. Clin. Expt. Immunol., 47, 635-644.

Chin, W. and Collins, W.E. (1980). Comparative studies of three strains of P. falciparum isolated by the culture method of Trager and Jensen. Am. J. Trop. Med. Hyg., 29, 1143-1146.

Chin, W., Moss, D. and Collins, W.E. (1979). The continuous cultivation of P. fragile by the method of Trager and Jensen. Am. J. Trop. Med. Hyg., 28, 591-592.

Clark, I.A. (1978). Does endotoxin cause both the disease and parasite death in acute Malaria and Babesia, Lancet, 2, 75-77.

Clark, I.A. (1979). Resistance to Babesia Spp. and Plasmodium Spp. in mice pretreated with an extract of Coxiella burnetti. Infect. Immun., 24, 319-325.

Clark, I.A. and Allison, A.C. (1974). Babesia microti and Plasmodium berghei yoelii infections in nude mice. Nature, 259, 328-329.

Clark, I.A. and Hunt, N.H. (1983). Evidence for reactive oxygen intermediates causing haemolysis and parasite death in malaria. Infect. Immun., 39, 1-6.

Clark, I.A., Allison, A.C., Cox, F.E.G. (1976). Protection of mice against Babesia and Plasmodium with BCG. Nature, 259, 309-311.

Cohen, S. (1982). Progress in malaria vaccine development. Br. Med. Bull. 38. 161-165.

Clark, I.A., Cox, F.E.G. and Allison, A.C. (1977). Protection of mice against Babesia Spp. and Plasmodium Spp. with killed Corynebacterium parvum. Parasitology, 74, 9-18.

Clark, I.A., Virelizier, J.L., Carswell, E.A. and Wood, P.R. (1981). Possible importance of macrophage derived mediators in acute malaria. Infect. Immun., 32, 1058-1066.

Clyde, D.F., McCarthy, V.C., Miller, R.M. and Woodward, W.E. (1975). Immunization of man against falciparum and vivax malaria by use of altenuated sporozoites. Am. J. Trop. Med. Hyg., 24, 397-401.

Cochrane, A.H., Nussenzweig, R.S. and Nardin, E.H. (1980). Immunization against sporozoites P. 163-202, in J.P. Kreier ed., Malaria, Third edn. Academic Press, New York.

Cochrane, A.H., Santoro, F., Nussenzweig, V., Gwadz, R.W. and Nussenzweig, R.S. (1982). Monoclonal antibodies identify the protective antigens of sporozoites of P. knowlesi. Proc. Natl. Acad. Sci. USA, 79, 5651-5655.

Cohen, S. and Butcher, G.A. (1970). Properties of protective malarial antibody. Immunology, 19, 369-383.

Cohen, S. and Butcher, G.A. (1971). Serum antibody in acquired malarial immunity. Trans. R. Soc. Trop. Med. Hyg., 65, 125-135.

Cohen, S. and McGregor, I.A. (1963). In: Symposium on immunity to parasitic diseases. Eds. Garnham, P.C.C., Pierce, A.C. and Roitt, I. Oxford, Blackwell.

Cohen, S., Butcher, G.A. and Crandall, R.B. (1969). Action of malarial antibody in vitro. Nature, 223, 368-371.

Cohen, S., McGregor, A., and Carrington, S. (1961). Gamma-globulin and acquired immunity to human malaria. Nature, 192, 733-737.

Coleman, R.M., Bruce, A. and Rencricca, N.J. (1976). Malaria: Macrophage migration inhibition factor (MIF). J. Parasitol., 62, 137-138.

Collins, W.E., Anders, R.F., Pappaioanou, M., Campbell, G., Brown, G.V., Kemp, D., Coppel, R., Skinner, J.C., Andrysiak, P.M., Favaloro, J.M., Carcoron, L.M., Broderson, J.R., Mitchell, G.F. and Campbell, C.C. (1986). Immunization of Aotus monkeys with recombinant proteins of an erythrocyte surface antigen of P. falciparum. Nature, 323, 259-262.

Coppel, R.L., Cowman, A.F., Anders, R.F., Bianco, A.E., Saint, R.B., Lingelbach, K.R., Kemp, D.J. and Brown, G.V. (1984). Immune sera recognise on erythrocytes a P. falciparum antigen composed of repeated amino acid sequences. Nature, 310, 789-791.

Cottrell, B.J., Playfair, J.H.L. and De Souza, J.B. (1977). P. yoelii and P. vinckei. The effects of non specific immunostimulation on murine malaria. Expt. Parasitol., 43, 45-53.

Cowman, A.F., Coppel, R.L. and Saint, R.B. (1984). The RESA polypeptide of P. falciparum contains two separate blocks of tandem repeats encoding antigenic epitopes that are normally immunogenic in man. Mol. Biol. Med., 2, 207-22.

Dame, J.B., Williams, J.L., McCutchan, T.F., Weber, J.L., Wirtz, R.A., Hockmeyer, W.T., Maloy, W.L., Haynes, J.D., Schneider, I., Roberts, D., Sanders, G.S., Reddy, E.P., Diggs, C.L. and Miller, L.H. (1984). Structure of the gene encoding the

immunodominant surface antigen on the sporozoite of the human malaria parasite P. falciparum. Science, 225, 593-599.

Davies, P. and Bonney, R.J. (1979). Secretory products of mononuclear phagocytes: a brief review. J. Reticuloendothelial. Soc., 26, 37-41.

David, P.H., Hommel, M., Miller, L.H., Udeinya, I.J. and Olignio, L.D. (1983). Parasite sequestration in P. falciparum malaria: spleen and antibody modulation of cytoadherence of infected erythrocytes. Proc. Natl. Acad. Sci. USA, 80, 5075-5079.

Deans, J.A. (1984). Protective antigens of bloodstage P. knowlesi parasites. Phil. Trans. Roy. Soc. Lond., B307, 159-169.

Deans, J.A. and Cohen, S. (1983). Immunology of Malaria. Ann. Rev. Microbiol., 37, 25-49.

Deans, J.A., Alderson, T., Thomas, A.W., Mitchell, G.A., Lennox, E.S. and Cohen, S. (1982). Rat monoclonal antibodies which inhibit the in vitro multiplication of P. knowlesi. Clin. Expt. Immunol., 49, 297-309.

Diggs, C.L. and Ostler, A.G. (1969). Humoral immunity in rodent malaria 2. Inhibition of parasitaemia by serum antibody. J. Immunol., 102, 298-305.

Diggs, C.L. and Ostler, A.G. (1975). Humoral immunity in rodent malaria 3. Studies on the site of antibody action. J. Immunol., 114, 1243-1247.

Divo, A.A. and Jensen, J.B. (1982A). Studies on serum requirements for the cultivation of P. falciparum 2. Medium enrichment. Bull. World Health Organ. 60, (4), 571-575.

Divo, A.A. and Jensen, J.B. (1982B). Studies on serum requirements for the cultivation of P. falciparum 1. Animal sera. Bull. World Health Organ. 60, (4), 565-569.

Dluzewski, A. R., Ling, I. T., Rangachari, K., Bates, P. and Wilson, R. J. M. (1984). A simple method for isolating viable mature parasites of P. falciparum from cultures. Trans. R. Soc. Trop. Med. Hyg. 78. 622-637.

Druilhe, P., Jacquier, P., Lambert, D. and Gentilini, M. (1980). P. falciparum in-vitro culture : improvement using umbilical cord serum and medium modifications. Tropenmed. Parasit. 31. 409-413.

Druilhe, P., Mazier, D. Brandicourt, O. and Gentilini, M. (1983). One-step P. falciparum cultivation - application to in-vitro drug testing. Tropenmed. Parasit. 34, 233-234.

Dixon, G.J., Dolmadge, E.A. and Schabel, F.M. Jr. (1966). Growth requirements and biological characteristics of leukemia L1210 cells in culture. Cancer Chemother. Rep., 50, 247-254.

Dock rell, H. and Playfair, J.H.L. (1983). Killing of bloodstage murine malaria parasites by hydrogen peroxide. Infect. Immun., 39, 456-459.

Dock rell, H., De Souza, J.B. and Playfair, J.H.L. (1980). The role of the liver in immunity to blood stage murine malaria. Immunology, 41, 421-430.

Duszenko, M., Ferguson, M.A.J., Lamont, G.S., Rifkin, M.R. and Cross, G.A.M. (1985). Cysteine eliminates the feeder cell requirement for cultivation of Trypanosoma brucei bloodstream forms in vitro. J. Expt. Med., 162, 1256-1263.

Dvorak, J.A., Miller, L.H., Whitehouse, W.C. and Shiroishi, T. (1975). Invasion of erythrocytes by malaria merozoites. Science, 187, 748-750.

Ellis, J., Osaki, L.S., Gwadz, R.W., Cochrane, A.H., Nussenzweig, V., Nussenzweig, R.S. and Godson, G.N. (1983). Circumsporozoite geme of P. cynomalgi (Gombak) CDNA cloning and expression of the repetative CS epitope. Proc. Natl. Acad. Sci. USA., 81, 7520-7524.

Enea, V., Ellis, J., Zavala, F., Arnot, D.E., Asavanich, A., Masuda, A., Quakyi, I. and Nussenzweig, R.S. (1984). DNA cloning of P. falciparum circumsporozoite gene: amino acid sequence of repetitive epitope. Science, 225, 628-630.

Epstein, N., Miller, L.H., Kaushal, D.C., Udeinyz, I.J., Rener, J., Howard, R.J., Asofsky, R., Aikawa, M. and Hess, K.L. (1981). Monoclonal antibodies against a specific determinant on malaria (P. knowlesi) merozoites block erythrocyte invasion. J. Immunol., 127, 212-217.

Fenton, B., Walker, A. and Walliker, D. (1985). Protein variation in clones of P. falciparum detected by two-dimensional electrophoresis. Mol. Biochem. Parasitol., 16, 173-183.

Ferraroni, J.J. and Spear, C.A. (1982). Adoptive transfer of resistance to P. berghei with spleen cells and serum from fansidar cured mice. Infect. Immun., 36, 1109-1114.

Frankenburg, S., Londner, M.V. and Greenblatt, C.V. (1980). Cellular changes in the bone marrow of P. berghei infected mice. Cell. Immunol., 55, 185-190.

Garnham, P.C.C. (1966). Malaria parasites and other Haemosporidia. Blackwell Scientific Publications, Oxford.

Gauci, R., Bennet, D., Clark, I.A. and Bryant, C. (1982). The induction of tyrosine aminotransferase activity and its use as an indirect assay for endotoxin in mice infected with P. uinckei petteri. Int. J. Parasitol., 12, 279-284.

Golenser, J., Spira, D.T. and Zuckerman, A. (1975). Neutralizing antibody in rodent malaria. Trans. R. Soc. Trop. Med. Hyg., 69, 251-258.

Gravely, S.M. and Kreier, J.P. (1976). Adoptive transfer of immunity to P. berghei with immune T and B. lymphocytes. Infect. Immun., 14, 184-190.

Graves, P.M., Carter, R. and McNeil, K.M. (1984B). Gametocyte production in cloned lines of P. falciparum Am. J. Trop. Med. Hyg., 33, (6), 1045-1050.

Graves, P.M., Carter, R., Keystone, J.S. and Seely, D.C., (1984A). Drug sensitivity and isoenzyme type in cloned lines of P. falciparum. Am. J. Trop. Med. Hyg. 33, (2), 212-219.

Graves, P.M., Carter, R., Burkot, T.R., Rener, J., Kaushal, D.C. and Williams, J.L. (1985). Effects of transmission blocking monoclonal antibodies on isolates of P. falciparum. Infect. Immun., 48, 611-616.

Green, T.J. and Kreier, J.P. (1978). Demonstration of the role of cytophilic antibody in resistance to malaria parasites (P. berghei) in rats. Infect. Immun., 19, 138-145.

Green, T.J., Morhardt, M. Brackett, R.G. and Jacobs, R.L. (1981). Serum inhibition of merozoite dispersal from P. falciparum schizonts: indicator of immune status. Infect. Immun., 31, 1203-1208.

Green, T.J., Gadsden, G., Seed, T., Jacobs, R., Mordhardt, M. and Brackett, R. (1985). Cloning and characterisation of P. falciparum FCR/FMG strain. Am. J. Trop. Med. Hyg., 34, (1), 24-30.

Greenwood, B.M. and Vick, R.M. (1975). Evidence for a malaria mitogen in human malaria. Nature, 257, 592.

Greenwood, B.M. Herrick, E.M. and Holbarow, E.J. (1970). Speckled anti-nuclear factor in African sera. Clin. Expl. Immunol., 7, 75-83.

Greenwood, B.M., Muller, A.S. and Valkenburg, H.A. (1971). Rheumatoid factor in Nigerian sera. Clin. Expt. Immunol., 8, 161-173.

Greenwood, B.M., Oduloju, A.J. and Platts-Mills, T.A.E. (1979). Partial characterisation of a malaria mitogen. Trans. R. Soc. Trop. Med. Hyg., 73, 178-182.

Greenwood, B.M., Oduloju, A.J. and Stratton, D. (1977). Lymphocyte changes in acute malaria. Trans. Roy. Soc. Trop. Med. Hyg., 71, 408-410.

Grun, J.L., Weidanz, W.P. (1987). Cultivation of P. falciparum in commercially available sera depleted of natural antibodies reactive with human erythrocytes. J. Parasitol., 73, (2), 384-388.

Gwadz, R.W. (1976). Malaria: successful immunization against the sexual stages of P. gallinaceum. Science, 193, 715-725.

Hamburger, J. and Kreier, J.P. (1975). Antibody mediated elimination of malarial parasites (P. berghei) in vitro. Infect. Immun., 12, 339-345.

Harrison, G. (1978). Mosquitoes, Malaria and Man: a history of the hostilities since 1880. John Murray, London.

Haynes, J.D., Diggs, C.L., Hines, F.A. and Desjardins, R.E. (1976). Culture of human malaria parasites, P. falciparum. Nature, 263, 767-769.

Hempelmann, E., Ling, I. and Wilson, R.J.M. (1981). S antigens and isozymes in strains of P. falciparum. Trans. R. Soc. Trop. Med. Hyg., 75, 855-858.

Herrington, D.A., Clyde, D.F., Losonsky, G., Cortesia M., Murphy, J.R., Davis, J., Baqar, S., Felix, A.M., Heimer, E.P., Gillessen, D., Nardin, E., Nussenzweig, R.S., Nussenzweig, V., Hollingdale, M.R. and Levine, M. (1987). Safety and immunogenicity in man of a synthetic peptide malaria vaccine against P. falciparum sporozoites. Nature, 328, 257-259.

Hirumi, H., Doyle, J.J. and Hirumi, K. (1977). African trypanosomes: cultivation of animal infective T. brucei in vitro. Science, 196, 992-994.

Hirumi, H., Hirumi, K., Nelson, R.T. and Bwago, J.J. (1980). Present status of in vitro cultivation of animal-infective African trypanosomes, in the in vitro cultivation of the

pathogens of tropical diseases. Tropical diseases research series Nos 3, 165-200. Schwabe and Company, Basel, Switzerland.

Holder, A.A. and Freeman, R.R. (1984). Protective antigens of rodent and human bloodstage malaria. Phil. Trans. Roy. Soc. London., 303, 171-177.

Holder, A.A., Lockyer, M.J., Odink, K.G., Sandhu, J.S., Riveros-Moreno, V., Nicholls, S.C., Hillman, Y., Davey, L.S., Tizard, M.L.V., Schwartz, R.T. and Freeman, R.R. (1985). Primary structure of the precursor to the three major surface antigens of P. falciparum merozoites. Nature, 317, 270-273.

Hollingdale, M.R., Leland, P., Leaf, J.E. and Beaudoin, R.L. (1983). The influence of cell type and culture medium on the in vitro cultivation of exoerythrocytic stages of P. berghei. J. Parasitol. 69, p. 346-352.

Hollingdale, M.R. and Leaf, J.L. (1981). In vitro cultivation of the exoerythrocytic stages of P. berghei from sporozoites. Science, 213, 1021-1023.

Hollingdale, M.R., Collins, W.E., Campbell, C.C. and Schwartz, A.L. (1985). In vitro culture of two populations (dividing and non dividing) of exoerythrocytic parasites of P. vivax. Am. J. Trop. Med. Hyg., 34, (2), 216-222.

Hollingdale, M.R., Zavala, F., Nussenzweig, R.S. and Nussenzweig, V. (1982). Antibodies to the protective antigen of P. berghei sporozoites prevent entry into cultured cells. J. Immunol., 128, 1928-1930.

Hollingdale, M.R., Nardin, E.H., Tharavanij, S., Schwartz, A.L. and Nussenzweig, R.S. (1984). Inhibition of entry of P.

falciparum an P. vivax sporozoites into cultured cells: an in vitro assay of protective antibodies. J. Immunol., 132, 909-913.

Hommel, M. (1981). Malaria: Immunity and prospects for vaccination. West. J. Med., 135, 285-299.

Hommel, M., David, P.H. and Oligino, L.D. (1983). Surface alterations of erythrocytes in P. falciparum malaria. J. Expt. Med., 157, 1137-1148.

Howard, R.J., Marsh, K., Aley, S.B. and McBride, J. (1986B). Antigenic diversity of P. falciparum antigens in isolates from Gambian patients II. The schizont surface glycoprotein of Mr 200 000. Parasite Immunol., 8, 57-68.

Howard, R.J., Panton, L.J., Mars, K., Ling, I.T., Winchell, E.J. and Wilson, R.J.M. (1986A). Antigenic diversity and size diversity of P. falciparum antigens in isolates from Gambian patients I. S. antigens. Parasite Immunol., 8, 39-55.

Ifediba, T. and Vanderberg, J.P. (1980). Peptones and calf serum as a replacement for human serum in the cultivation of P. falciparum. J. Parasitol., 66, 236-239.

Ifediba, T. and Vanderberg, J.P. (1981). Complete in vitro maturation of P. falciparum gametocytes. Nature 194, 364-366.

Ishii, T., Hishinuma, I., Bannai, S. and Sugita, Y. (1981). Mechanism of growth promotion of mouse lymphoma L1210 cells in vitro by feeder layer or 2ME. J. Cell. Physiol., 107, 283-293.

Janse, C.J., Mons, B., Croon, J.A.B. and Van der Kay, H.J. (1984). Longterm in vitro cultures of P. berghei and preliminary observations on gametocytogenesis. Int. J. Parasitol., 14, 317-320.

Janse, C.J., Mons, B., Rouwenhorst, R.J., Van der Klooster, P.F.J., Overdulve, J.P. and Van der Kaay, H.J. (1985). In vitro formation of ookinetes and functional maturity of P. berghei gametocytes. Parasitology, 91, 19-29.

Jayawardena, A.N. Janeway, C.A. and Kemp, J.D. (1979). Experimental malaria in the CBA/N mouse. J. Immunol., 123, 2532-2539.

Jayawardena, A.N., Murphy, D.B., Jaenway, C.A. and Gershon, R.K. (1982). T. cell-mediated immunity in malaria. 1 The Ly phenotype of T cells mediating resistance to P. yoelii. J. Immunol., 129, 377-381.

Jayawardena, A.N. Targett, G.A.T., Leuchars, E. and Davies A.J.S. (1978). The immunological response of CBA mice to P. yoelii II. Passive transfer of immunity with serum and cells. Immunology, 34, 157-165.

Jayawardena, A.N., Targett, G.A.T., Davies, A.J.S., Leuchars, E. and Carter, R. (1975). The passive transfer of immunity to P. berghei yoelii. Trans. R. Soc. Trop. Med. Hyg., 69, 426.

Jensen, J.B. (1979). Observations on Gametogenesis in P. falciparum from continuous culture. J. Protozool., 26, 129-132.

Jensen, J.B., Boland, M.T. and Akood, M. (1982A). Induction of crisis forms in cultured P. falciparum by human serum from Sudan. Science, 216, 1230-1233.

Jensen, J.B., Boland, M. T. and Haynes, M. (1982B). P. falciparum: Rapid assay for in vitro inhibition due to human serum from residents of malarious areas. Expt. Parasitol. 54, 416-424.

Jensen, J.B., Capps, T.C. and Carlin, J.M. (1981). Clinical drug-resistant falciparum malaria acquired from cultured parasites. Am. J. Trop. Med. Hyg., 30, 523-525.

Jensen, J.B., Trager, W. and Doherty, J. (1979). P. falciparum; Continuous cultivation in a semi-automated apparatus. Expt. Parasitol., 48, 36-41.

Jensen, J.B., Boland, M.T., Allen, J.S., Carlin, J.M., Waa, J.A.V., Divo, A.A. and Akood, M.A.S. (1983A). Association between human serum induced crisis forms in cultured P. falciparum and clinical immunity to malaria in Sudan. Infect. Immun., 41, 1302-1311.

Jensen, M.D., Canley, M. and Dale Helstrowski, L. (1983B). Culture of P. falciparum The role of pH, glucose and lactate. J. Parasitol. 69 (6), 1060-1067.

Kaushal, D.C., Carter, R., Miller, L.H. and Krishna, G. (1980). Gametocytogeneeis by malaria parasites in continuous culture. Nature, 286, 490-492.

Kaushal, D.C., Carter, R., Rener, J., Grotendorst, C.A., Miller, L.H. and Howard, R.J. (1983). Monoclonal antibodies against surface determinants on gametes of P. gallinaceum block transmission of malaria parasites to mosquitoes. J. Immunol., 131, 2557-2567.

Kharazmi, A. and Jepsen, S. (1984). Enhanced inhibition of in vitro multiplication of P. falciparum by stimulated human polymorphonuclear leukocytes. Clin. Expt. Immunol., 57, 287-292.

Khusmith, S., Druilhe, P. and Gentilini, M. (1982). Enhanced P. falciparum phagocytosis by monocytes from immune individuals. Infect. Immun., 35, 874-879.

Kitchen, S.F. (1939). The infection of reticulocytes by P. vivax. Am. J. Trop. Med. Hyg., 18, 347-353.

Knowles, B.B., Howe, C.C. and Arden, D.P. (1980). Human hepatoma carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. Science, 209, 497-499.

Krotoski, W.A., Collins, W.E., Bray, R.S., Garnham, P.C.C., Cogswell, F.B., Gwadz, R.W., Killick-Kendrick, R., Wolf, R., Sinden, R., Koontz, L.C. and Stanfill, D.S. (1982). Demonstration of hypnozoites in sporozoite transmitted P. vivax infection. Am. J. Trop. Med. Hyg., 31, 1291-1293.

Krotoski, W.A., Garnham, P.C.C., Bray, R.S., Krotoski, D.M., Killick-Kendrick, R., Draper, C.C., Targett, G.A.T. and Guy, M.M. (1982A). Observations on early and late post sporozoite tissue stages in primate malaria I. Discovery of a new latent form of P. cynomolgi (the hyprnozoite) and failure to detect hepatic forms within the first 24 hours after infection. Am. J. Trop. Med. Hyg., 31, 24-35.

Krotoski, W.A., Collins, W.E., Bray, R.S., Garnham, P.C.C., Cogswell, F.B., Gwadz, R.W., Killick-Kendrick, R., Wolf, R., Sinden, R., Zoontz, L.C. and Stanfill, P.S. (1982B). Observations on early and late post sporozoite tissue stages in primate malaria II. The hypnozoite of P. cynomolgi bastianelli from 3 to 105 days after infection and detection of 36 to 40 hour pre erythrocytic forms. Am. J. Trop. Med. Hyg. 131, 211-225.

Kyle-Webster, H., Thaithong, S., Pavanand, K., Yongvanitchit, K., Pinswasdi, C. and Boudreau, G.F. (1985). Cloning and characterisation of mefloquine-resistant P. falciparum from Thailand. Am. J. Trop. Med. Hyg. 3, (6), 1022-1027.

Lambros, C. and Vanderberg, P. (1979). Synchronization of P. falciparum erythrocytic stages in culture. J. Parasitol. 65, (3), 418-420.

Langreth, S.G., Reese, R.T., Motyl, M.R. and Trager, W. (1979). P. falciparum: Loss of knobs on the infected erythrocyte surface after long term cultivation. Expt. Parasitol., 48, 213-219.

Lanners, H.N. and Trager, W. (1984). Comparative infectivity of knobless and knobby clones of P. falciparum in splenectomised and intact Aotus trivirgatus monkeys. Z. fur. Parasiten., 70, 739-745.

Larrong, G., Magnoval, J.F. and Morro, F. (1981). A propos de l'obtention par culture in vitro de formes intra-erythrocytaires e P. vivax. C.R. Acad. Sci. (Paris), 292, 929-390.

Laveran, C.L.A. (1880). A newly discovered parasite in the blood of patients suffering from malaria. Parasite etiology of attacks of malaria. Bull. Acad. Med., 19., 1235-1236.

Lehninger, A.L. (1982). Principals of Biochemistry. Worth Publishers Inc. New York, 913-941.

Luse, S.A. and Miller, L.H. (1971). Ultrastructure of parasitised erythrocytes in cardiac vessels. Am. J. Trop. Med. Hgy., 20, 655-660.

MacGregor, R.R., Sheagren, J.N. and Wolff, S.M. (1969). Endotoxin induced modification of P. berghei infection in mice. J. Immunol., 102, 131-138.

Manson, P. (1898). Surgeon Major Ronald Ross's recent investigations on the mosquito malaria theory, Br. Med. J. 1575-1577.

Marchiafava, E. and Celli, A. (1883). Sulle alterazione dei globuli rossi nella infezione della malaria e sulla genese della malanemia". Mem. R. Acad. Linci. Ser. 3, 18, 381-401.

Marsh, K. and Howard, R.J. (1986). Antigens induced on erythrocytes by P. falciparum: Expression of diverse and conserved determinants. Science, 231, 150-153.

Martin, L.K., Einheber, A., Sadun, E.H. and Wren, R.E. (1967). Effect of bacterial endotoxin on the course of P. berghei infection. Expt. Parasitol., 20, 186-199.

Matthews, N. (1981). Production of an anti-tumour cytotoxin by human monocytes. Immunology, 44, 135-142.

Mazier, D., Landau, I., Druilhe, P., Miltgent, F., Guguen-Guillouzo, C., Baccon, D., Baxter, J., Chigot, J.P. and Gentilini, M. (1984). Cultivation of the liver forms of P. vivax in human hepatocytes. Nature, 307, 367-369.

Mazier, D., Druilhe, P., Guguen-Guillouzo, C., Bayard, P., Soeun, V., Datry, A. and Gentilini, M. (1984). Hepatocytes as feeder layers for in vitro cultivation of P. falciparum blood stages. Trans. R. Soc. Trop. Med. Hyg., 78, 330-334.

Mazier, D., Landau, I., Miltgen, F., Druilhe, P., Lambiotte, M., Baccam, D. and Gentilini, M. (1982). Infestation in vitro d'heptocytes de Thamnomys adulte par des sporozoites et liberation de merozoites infestans. C.R. Acad. Sci. (Paris) 294, (3), 963-65.

McBride, J.S., Newbold,, C.I. and Anand, R. (1985). Polymorphisms of a high molecular weight schizont antigen of the human malaria parasite P. falciparum. J. Expt. Med., 161, 160-180.

McBride, J.S., Walliker, D. and Morgan, G. (1982). Antigenic diversity in the human malaria parasite P. falciparum. Science, 217, 254-257.

McDonald, V. and Phillips, R.S. (1978). P. chabaudi in mice: adoptive transfer of immunity with enriched populations of spleen T and B lymphocytes. Immunology, 34, 821-830.

McGregor, I.A. (1972). Immunology of malarial infection and its possible consequences. Br. Med. Bull., 28, 22-27.

McGregor, I.A., Gillies, H.M., Walters, J.H., Davies, A.H. and Pearson, F.A. (1956). Effects of heavy and repeated malarial infections on Gambian infants and children. Br. Med. J., 2, 686-692.

Michalopoulas, G., Russel, F. and Biles, G. (1979). Primary cultures of hepatocytes on human fibroblasts. In vitro, 15, 796-806.

Miller, L.H., Aikawa, M. and Dvorak, J.A. (1975). Malaria (P. knowlesi) merozoites: immunity and the surface coat. J. Immunol., 114, 1237-1242.

Miller, L.H., McAuliffe, F.M. and Mason, S.J. (1977). Erythrocyte receptors for malaria merozoites. Am. J. Trop. Med. Hyg., 26, 204-208.

Miller, L.H., Powers, K.G. and Shiroishi, T. (1977). P. knowlesi: functional immunity and antimerozoite antibodies in rhesus monkeys after repeated infections. Expt. Parasitol., 41, 105-111.

Miller, L.H., Mason, S.J., Dvorak, J.A., McGuinniss, M.H. and Rathman, I.K. (1975). Erythrocyte receptors fro (P. knowlesi) malaria: Duffy blood group determinants. Science, 189, 561-562.

Mitchell, G.H., Butcher, G.A. and Cohen, S. (1975). Merozoite vaccination against P. knowlesi malaria. Immunol., 29, 297-407.

Mitchell, G.H., Butcher, G.A. and Cohen, S. (1976). A note on the rapid development of P. falciparum gametocytes in vitro. Trans. R. Soc. Trop. Med. Hyg., 70, 12-13.

Mons, B., Nguyn Dinh, P., Croon, J.J.A.B., Van der Star, W. and Van der Kaay, H.J. (1981). Growth and invasion of P. vivaa in vitro. Poster presentation Spring Meeting British Society for Parasitology

Moore, G.E., Gerner, R.E. and Franklin, H.A. (1967). Culture of normal human leukocytes. J. Am. Med. Assocn., 199, 519-524.

Morgan, D.M.L. and Christenson, J. (1983). Polyamine oxidation and the killing of intracellular parasites. In. Advances in polyamine Research 4. Bachrach, U., Kaye, A.M. and Chayen, R. Eds. New York, Raven Press, 169.

Motyl, M.R. and Reese, R.T. (1983). P. falciparum: comparison of in vitro growth of knobby and knobless isolates. Am. J. Trop. Med. Hyg., 32, 226-236.

Nardin, E.H., Nussenzweig, V., Nussenzweig, R.S., Collins, W.E., Hainasuta, K.T., Tapchaisri, P. and Chomcharn, Y. (1982). Circumsporozoite proteins of human malaria parasites P. falciparum and P. vivax. J. Expt. Med., 154, 1225-1236.

Nathan, C.F. and Terry, W.D. (1975). Differential stimulation of murine lymphoma growth in vitro by normal and BCG activated macrophages. J. Expt. Med., 142, 887-902.

Nguyen-Dinh, P., Campbell, C.C. and Collins, W. (1980). Cultivation in vitro of the quartan malaria parasite P. inuii. Science, 209, 1249-1251.

Nguyen-Dinh, P., Gardner, A.L., Campbell, C.C., Skinner, J.C. and Collins, W.E. (1981). Cultivation in vitro of the vivax-type malaria parasite P. cynomolgi. Science, 212, 1146-1148.

Nussenzweig, R.S. (1967). Increased non-specific resistance to malaria produced by administration of killed Corynebacterium parvum. Expt. Parasitol. 21, 224-231.

Nussenzweig, R.S., Vanderberg, J., Most, H. and Orton, C. (1969). Specificity of protective immunity produced by X-irradiated P. berghei sporozoites. Nature, 222, 488-489.

Ockenhouse, C.F. and Shear, H.L. (1984). Oxidative killing of the intra erythrocytic malaria parasite P. yoelii by activated macrophages. J. Immunol., 132, 424-431.

Ockenhouse, C.F., Schulman, S. and Shear, H.L. (1984). Induction of crisis forms in the human malaria parasite P. falciparum by interferon activated, monocyte derived macrophages. J. Immunol., 133, 1602-1608.

Ohomori, H. and Yamamoto, I. (1982). Mechanism of augmentation of the antibody response in vitro by 2-mercaptoethanol in murine lymphocytes. J. Expt. Med., 155, 1277-1286.

Ojo-Amaize, E.A., Salimonu, L.S., Williams, A.I.O. (1981). Positive correlation between degree of parasitaemia, interferon titres and natural killer cell activity in P. falciparum infected children. J. Immunol. 127, 2296-2300.

Omata, Y., Yagami, K., Takei, K., Suzuki, N. and Nakabayashi, T. (1981). Protective reaction against malaria infection in mice sensitised with frozen and thawed Toxoplasma tachzoites. Z. fur. Bact. Mikrolo. Hyg. Med., 250, 223-235.

Ozaki, L.S., Svec, P., Nussenzweig, R.S. Nussenzweig, V. and Godson, G.N. (1983). Structure of the P. knowlesi gene coding for the circumsporozoite protein cell 34, 815-822.

Patarroyo, M.E., Romero, P., Torres, M.L., Clavijo, P., Moreno, A., Martinez, A., Rodriguez, R., Guzman, F. and Cabezas, E. (1987). Induction of protective immunity against experimental infection with malaria using synthetic peptides. Nature, 328, 629-632.

Perkins, M. (1981). Inhibitory effect of erythrocyte membrane proteins on the in vitro invasion of the human malarial parasite (P. falciparum) into its host cell. J. Cell. Biol., 90, 563-567.

Phillips, R.S. (1970). P. berghei: Passive transfer of immunity by antisera and cells. Expt. Parasitol., 27, 479-495.

Phillips, R.S. and Jones, V.E. (1972). Immunity to P. berghei in rats: maximum levels of protective antibody are associated with eradication of infection. Parasitology, 64, 117-127.

Phillips, R.S. and Wilson, R.J.M. (1978). Cryopreservation of P. falciparum in liquid nitrogen. Trans. R. Soc. Trop. Med. Hyg., 72, 643-644.

Phillips, R.S., Wilson, R.M. and Pasvol, G. (1976). Differentiation of gametocytes of P. falciparum in vitro. Trans. R. Soc. Trop. Med. Hyg., 70, 286.

Phillips, R.S., Wilson, R.J.M. and Pasvol, G. (1978). Differentiation of gametocytes in microcultures of human blood infected with P. falciparum. J. Protozool., 25, (3), 394-398.

Phillips, R.S., Trenholme, K.R., MacDougall, L.M., Pyott, A., Mclean, S. and Walker, E. (1987). Adaptation of P. falciparum to continuous culture using human and murine feeder cells. Med. Sci. Res., 15, 1167-1168.

Playfair, J.H.L. (1982). Immunity to malaria. Br. Med. Bull., 83, (2), 153-159.

Playfair, J.H.L. and DeSouza, J.B. (1979). Antibody responses in mice protected against malaria by vaccination. Parasite Immunol., 1, 197-208.

Potocnjak, P., Yoshida, N., Nussenzweig, R.S. and Nussenzweig, V. (1980). Monovelent fragments (Fab) of monoclonal antibodies to a sporozoite surface antigen (Pb 44) protect mice against malarial infection. J. Expt. Med., 151, 1504-1513.

Ponnudurai, T., Meuwissen, J.H.E., Leeuwenberg, A.D.E.M., Verhave, J.P. and Lensen, A.H.W. (1982). The production of mature gametocytes of P. falciparum in continuous cultures of different isolates infective to mosquitoes. Trans. R. Soc. Trop. Med. Hyg., 76, 242-250.

Puck, T. and Marcus, P.I. (1955). A rapid method for viable cell titration and clone production with Hela cells in tissue culture: The use of X-irrediated cells to supply conditioning factors. Proc. Natl. Acad. Sci. USA, 41, 432-437.

Rai-chowdhury, A.N. Chowdery, D.S. and Regis, M.L. (1979). Simultaneous propagation of P. melanae and P. falciparum in a continuous culture. Indian J. Med. Res., 70, 72-78.

Reese, R.T. and Motyl, M.R. (1979). Inhibition of in vitro growth of P. falciparum 1. effects of immune serum and purified immunoglobulin from owl monkeys. J. Immunol., 123, 1894-1899.

Reese, R.T., Trager, W., Jensen, J.B., Miller, D.A. and Tantravahi, (1978). Immunization against malaria with antigen from P. falciparum cultivated in vitro. Proc. Natl. Acad. Sci. USA. 115, 5665-5668.

Renapurkar, D.M. Pradhan, V.R., Sutar, N.K., Shirodkar, M., Sharma, K.D., Ajgaonkar, V.S. and Marathe, S.N. (1983). The

continuous in vitro cultivation of P. vivax. ICRS Med. Sci., 11, 7-8.

Rener, J., Carter, R., Rosenberg, Y. and Miller, L.H. (1980). Antigamete monoclonal antibodies synergistically block transmission of malaria by preventing fertilization in the mosquito. Proc. Nalt. Acad. Sci. USA, 77, 6797-6799.

Rener, J., Graves, P.M. Carter, R., Williams, J.L. and Burkat, T.R. (1983). Target antigens of transmission blocking immunity on gametes of P. falciparum. J. Expt. Med., 158, 976-981.

Rheinwold, I.G. and Green, H. (1975). Serial cultivation of strains of human epidermal keratinocytes; the formation of keratinizing colonies from single cells. Cell, 6, 331-334.

Roberts, D.W. and Weidanz, W.P. (1979). T-cell immunity to malaria in the B cell defficient mouse. Am J. Trop. Med. Hyg., 28, 1-3.

Roberts, D.W., Rank, R.G., Weidanz, W.P. and Finerty, J.F. (1977). Prevention of recrudescent malaria in nude mice by thymic grafting or treatment with hyper immune serum. Infect. Immun., 16, 821-826.

Ronai, Z., Avraham, H. and Sulitzeanu, D. (1981). Autoantibodies to red blood cells in rats infected with P. berghei. J. Parasitol., 67, 351-355.

Rosales-Ronquillo, M.C. and Silverman, P.H. (1974). In vitro ookinete development of the rodent malarial parasite, P. berghei. J. Parasitol., 60, 819-824.

Rosario, V. (1981). Cloning of naturally occurring mixed infections of malaria parasites. Science, 212, 1037-1038.

Rosenberg, E.B., Strickland, G.T., Yong, S. and Whalen, G.E. (1973). IgM antibodies to red cells and autoimmune anaemia in patients with malaria. Am. J. Trop. Med. Hyg., 22, 146-152.

Row, R. (1928). Observations sur le paludisme et la formation des gametes de Plasmodium en cultures. Bull. Soc. Path. Exot., 21, 607-610.

Sanderson, A., Walliker, D. and Molez, J.F. (1981). Enzyme typing of P. falciparum from Africa and some other Old World countries. Trans. R. Soc. Trop. Med. Hyg., 875, 2 63-267.

Sax, L.H., Reichman, K.H. (1980). Use of rabbit serum in the cultivation of P. falciparum. J. Parasitol, 66, 621-624.

Scheibel, L.W., Ashton, S.H. and Trager, W. (1979). P. falciparum: Microaerophilic requirements in human red blood cells. Expt. Parasitol., 47, 410-418.

Schmidt, J.A., Udeinya, I.J., Leech, J.H., Hay, R.J., Aikawa, M., Barnwell, J., Green, I. and Miller, L.H. (1982). P. falciparum malaria. an amelanotic cell line bears receptors for the knob ligand on infected erythrocytes. J. Clin. Invest., 70, 379-386.

Seed, T.M. and Kreier, J.P. (1980). In Kreier, J.P. Ed. Malaria Vol. 2. Pathology, vector studies and culture 1-46. Academic Press, New York.

Shaffer, J.G., Sienkiewicz, H.S. and Washington, J.E. (1953). The propagation of Entamoeba histolytica in tissue bearing culture without accompanying bacteria or other micro-organisms. Am. J. Hyg., 57, 366-379.

Shaper, A.G., Kaplan, M.I., Mody, N.J. and McIntyre, P.A. (1968). Malarial antibodies and auto antibodies to heart and other tissues in the immigrant and indigenous peoples of Uganda. Lancet, 1, 1342-1347.

Sherman, I.W. (1979). Biochemistry of Plasmodium (Malaria parasites) Microbial. Rev., 43, 453-495.

Sherman, I.W. and Tangoshi, L. (1974). Incorporation of ^{14}C-Amino acids by Malarial plasmodia (P. lophrae) VI changes in the kinetic constants of amino acid transport during infection. Expt. Parasitol. 35, 369-373.

Shortt, H.E. (1948). The life cycle of P. cynomolgi in its insect and mammalian hosts. Trans. R. Soc. Trop. Med. Hyg. 42, 227-230.

Shortt, H.E. and Garnham, P.C.C. (1948). Demonstration of a persisting exo-erythrocytic cycle in P. cynomolgi and its bearing on the production of relapses. Trans. R. Soc. Trop. Med Hyg. 1 1225-1232.

Shortt, H.E., Garnham, P.C.C., Covell, G. and Shute, P.G. (1948). The pre-erythrocytic stage of human malaria p. vivax Br. Med. J., 1, 1225-32.

Shortt, H.E., Fairley, N.H., Covell, G., Shute, P.G. and Garnham, P.C.C. (1951). The pre-erythrocytic stage of P. falciparum. Trans. R. Soc. Trop. Med. Hyg., 44, 405-419.

Sinden, R.E. and Smith, J.E. (1980). Culture of the liver stages (exoerythrocytic schizonts) of rodent malaria parasites from sporozoites in vitro. Trans. R. Soc. Trop. Med. Hyg., 74, 134-136.

Sinden, R.C. and Smith, J.E. (1982). The role of the Kupffer cell in the infection of rodents by sporozoites of Plasmodium: uptake of sporozoites by perfused liver and establishment of infection in vitro. Acta Trop., 39, 11-27.

Sinden, R.E. and Strong K. (1978). An ultrastructural study of the sporogonic development of P. falciparum in Anopheles gambiae. Trans. R. Soc. Trop. Med. Hyg., 72, 477-491.

Sinden, R.E., Hartley, R.H. and Winger, L. (1985). The development of Plasmodium ookinetes in vitro: an ultrastructural study of meiotic division. Parasitology 91, 227-244.

Smalley, M.E. (1976). P. falciparum gametocytogenesis in vitro. Nature, 264, 271-272.

Smith, G.L., Godson, G.N., Nussenzweig, V., Nussenzweig, R.S., Barnwell, J.W. and Moss, B. (1984). P. knowlesi sporozoite antigen: expression by infecti recombinant Vaccine virus. Science, 224, 297-399.

Smith, J.E., Sinden, Beadle, J. and Hartley (1981). The effect of silica treatment on the uptake and infectivity of P. yoelii sporozoites in vitro and in vivo. Trans. R. Soc. Trop. Med. Hyg., 75, 605-606.

Smith, J.E., Meis, J.F.G.M., Ponndurai, T., Verhave, J.P. and Moshage, H.J. (1984). In vitro culture of exoerythrocytic forms of P. falciparum in adult human hepatocytes. Lancet, 2, 757-758.

Sulzer, A.J. and Latorre, C.R. (1977). A simplified method for in vitro production of schizonts of primate malarias useful as antigen in serologic tests. Trans R. Soc. Trop. Med. Hyg., 71, 553-554.

Spira, D.T., Silverman, P.H. and Gaines, C. (1970). Anti-thymocyte serum effects on P. berghei. infections in rats. Immunology, 19, 759-766.

Spitalny, G.L. and Nussenzweig, R.S. (1973). P. berghei: Relationship between protective immunity and anti-sporozoite (CSP) antibody in mice. Expt. Parasit, 33, 168-178.

V PTO

Sulzer + Lafone (1977)

Strome, C.P.A., De Sentis, P.L. and Beaudoin, R.L. (1979). The cultivation of the exoerythrocytic stages of P. berghei from sporozoites. In vitro, 15, 531-536.

Tait, A. (1981). Analysis of protein variation in P. falciparum by two dimensional gel electrophoresis. Mol. Biochem. Parasitol., 2, 205-218.

Talioferro, W.H. (1929). The immunology of parasitic infection. Century. New York.

Tanner, M. (1980). Studies on the mechanisms supporting the continuous growth of Trypanosoma (Trypanozoon) brucei as blood steam-like forms in vitro. Acta Trop., 37, 203-220.

Tanapat, P., Gaetjens, E. and Broome, J.D. (1978). Growth promoting and inhibitory activities of T3T and other cell lines for thiol dependent lymphoma cells in vitro. Proc. Soc. Expt. Biol. Med., 157, 516-520.

Targett, G.A.T. and Voller, A. (1965). Studies on antibody levels during vaccination of Rhesus monkeys against P. knowlesi. Br. Med. J., 2, 1104-1106.

Taverne, J., Dockerell, H.M. and Playfair, J.H.L. (1981). Endotoxin induced serum factor kills malaria parasites in vitro. Infect. Immun., 33, 83-89.

Thaithong, S. and Beale, G.H. (1981). Resistance of ten Thai isolates of P. falciparum to chloroquine and pyrimethamine by in vitro tests. Trans. R. Soc. Trop. Med. Hyg., 75, 271-273.

Thaithong, S., Beale, G.H. and Chatmongkonkul, M. (1983). Susceptibility of P. falciparum to five drugs: an in vitro study of isolates mainly from Thailand. Trans. R. Soc. Trop. Med. Hyg., 77, 228-231.

Thaithong, S., Sublinwong, T. and Beale, G.H. (1981). Enzyme typing of some isolates of P. falciparum from Thailand. Trans. R. Soc. Trop. Med. Hyg., 75, 268-270.

Thaithong, S., Beale G.H., Fenton, B., McBride, J., Rosario, V., Walker, A. and Walliker D. (1984). Clonal diversity in a single isolate of the malaria parasite P. falciparum. Trans. R. Soc. Trop. Med. Hyg., 78, 242-245.

Toohey, J.I. (1975). Sulfhydryl dependence in primary explant hemapoetic cells. Inhibition of growth in vitro with vitamin B12 compounds. Proc. Natl. Acad. Sci. USA., 72, 73-77.

Trager, W. (1971). A new method for intraerythrocytic cultivation of malaria parasites. (P. coatneyi and P. falciparum). J. Protozool., 18, 239-242.

Trager, W. (1982). Cultivation of malaria parasites. Br. Med. Bull., 38, 129-131.

Trager, W. and Jensen, J.B. (1976). Human malaria parasites in continuous culture. Science, 193, 673-675.

Trager, W. and Jensen, J.B. (1977). P. falciparum in culture: use of outdated erythrocytes and description of the candle jar method. J. Parasitol., 63, 883-886.

Trager, W. and Jensen, J.B. (1978). Cultivation of malaria parasites. Nature 273, 621-622.

Trager, W., Tershakov, M., Lyandvert, L., Stanley, H., Lanners, N. and Gubert, E. (1981). Clones of the malaria parasite P. falciparum obtained by microscopic selection and their characterisation with regard to knobs, chloroquine sensitivity and formation of gametocytes. Proc. Natl. Acad. Sci. USA, 78, 6527-6530.

Troye-Blomberg, M., Perlmann, H., Patarrayo, M.E. and Perlmann, P. (1983B). Regulation of the immune response in P. falciparum malaria II. Antigen specific proliferative responses in vitro. Clin. Expt. Immunol., 53, 345-352.

Troy-Blomberg, M., Romero, P., Patarroyo, M.E., Bjorkman, A. and Perlman, P. (1984). Regulation of the immune response in P. falciparum malaria III. Proliferative response to antigen in vitro and subset composition of T cells from patients with acute infections or from immune donors. Clin. Expt. Immunol., 58, 380-386.

Troy-Blomberg, M., Sjoholm, P.E., Perlmann, H., Patarroyo, M.E. and Perlman, P. (1983A). Regulation of the immune response in P. falciparum malaria I. Non specific proliferative responses in vitro and characterisation of lymphocytes. Clin. Expt. Immunol., 53, 335-344.

Udeinya, I.J., Graves, P.M., Carter, I.R., Aikawa, M. and Miller (1983A). P. falciparum: Effect of time in continuous culture on Binding to human endothelial cells and a melanotic melanoma cells. Expt. Parasitol., 56, 207-214.

Udeinya, I.J., Graves, P.M., Carter, I.R., Aikawa, M. and Miller (1983)B). P. falciparum strain specific antibody blocks binding of infected erythrocytes to amelonatic melanoma cells. Nature, 303, 429-431.

Vanderberg, J.P. (1975). Development of infectivity by the P. berghei sporozoite. J. Parasitol., 61, 43-50.

Vanderberg, J.P., Nussenzweig, R.S. and Most, H. (1969). Protective immunity produced by the injection of x irradiated sporozoites on P. berghei V. In vitro effects of immune serum on sporozoites. Mil. Med., 134, 1183-1190.

Vanderberg, J.P., Weiss, M.M. and Mack, S.R. (1975). In vitro cultivation of sporogonic stages of Plasmodium: a review. Bull. World. Health Organ., 55, 377-392.

Wahlgren, M., Berzins, K., Perlman, P. and Bjorkman, A. (1983). Characterisation of the humoral immune response in P. falciparum malaria I. Estimation of antibodies to P. falciparum or human erythrocytes by means of micro ELISA. Clin. Expt. Immunol., 54, 127.

Walliker, D. (1983). The genetic basis of diversity in malaria parasites. Adv. Parasitol., 22, 217-259.

Warrell, D.A. (1987). Pathophysiology of severe falciparum malaria in man. Parasitology, 94, 553-576.

Weinbaum, F.I., Evans, C.B. and Tigelaar, R.E. (1976). Immunity to P. berghei yoelii in mice I. The course of infection in T cell and B cell deficient mice. J. Immunol., 117, 1999-2005.

Weiss, M.M. and Vanderberg, J.P. (1977). Studies on Plasmodium ookinetes II in vitro formation of P. berghei ookinetes. J. Parasitol, 63, 923-924.

Wells, D.E. and Price, P.J. (1983). Simple rapid methods for freezing hybridomas in 96 well microculture plates. J. Immunol. Methods., 59, 49-52.

Wells, R.A., Pavanand, K., Zolyomi, S., Permpanich, B. and McDermott, R.P. (1979). Loss of circulating T. lymphocytes with normal levels of B and 'null' lymphocytes in Thai adults with malaria. Clin. Expt. Immunol., 35, 202-209.

Wells, R.A., Pavanand, K., Zolyomi, S., Permpanich, B. and McDermott, R.P. (1980). Anti-lymphocyte antibodies in sera of Thai adults infected with P. falciparum or P. vivax. Clin. Expt. Immunol., 39, 663-670.

Wickham, J.M., Dennis, E.D. and Mitchell, G.H. (1980). Long term cultivation of a simian malaria parasite (P. knowlesi) in a semi automated apparatus. Trans. R. Soc. Trop. Med. Hyg., 74, 789-792.

Willet, G.P. and anfield, C.J. (1984). P. falciparum: continuous cultivation of erythrocytic stages in plasma free culture medium. Expt. Parasitol., 57, 76-80.

Wilson, R.J.M. (1980). Serotyping P. falciparum malaria with Stantigens. Nature, 284, 451.

Wilson, R.J.M., McGregor, I.A. and Williams, K. (1975). Occurrence of S antigens in serum in P. falciparum infections in man. Trans. R. Soc. Trop. Med. Hyg. 69, 453-459.

Wilson, R.J.M., McGregor, I.A., Hall, P., Williams, K. and Bartholomew, R. (1969). Antigens associated with P. falciparum infections in man. Lancet, 2, 201-205.

Winchell, E.J., Ling, I.T. and Wilson, R.J.M. (1984). Metabolic labelling and characterisation of S antigens, the heat-stable, strain specific antigens of P. falciparum. Mol. Biochem. Parasitol., 10, 287.

W.H.O. (1965). Technical Report Series, 296, 29.

W.H.O. (1985). Weekly Epidemiological Record, 60, 337-344.

Wyler, D.J. (1976). Peripheral lymphocyte subpopulations in human falciparum malaria. Clin Expt. Immunol., 23, 471-476.

Wyler, D.J. and Gallin, J.I. (1977). Spleen derived mononuclear cell chemotactic factor in malaria infections: a possible mechanism for splenic macrophage accumulation. J. Immunol., 118, 478-484.

Yoshida, N., Nussenzweig, R.S., Potocnjak, P., Nussenzweig, V. and Aikawa, M. (1980). Hybridoma induces protective antibodies directed against the sporozoite stage of malaria parasites. Science, 207, 71-73.

Young, J.F., Hockmeyer, W.T., Gross, M., Ballou, W.R., Wirtz, R.A., Trosper, J.H., Beaudin, R.L., Hollingdale, M.R., Miller, L.H., Diggs, C.L. and Rosenberg, M. (1985). Expression of P. falciparum circumsporozoite proteins in Escherichia coli for potential use in a human vaccine. Science, 228, 958-962.

Zavala, F. Tam, J.P., Hollingdale, M.R., Cochrane, A.H., Quakyi, I., Nussenzweig, R.S. and Nussenzweig, V. (1985). Rationale for development of a synthetic vaccine against P. falciparum malaria. Science, 228, 1436-1440.

Zouali, M., Druilhe, P., Gentilini, M. and Eyquem, A. (1982). High titres of anti-T antibodies and other haemagglutinins in human malaria. Clin. Expt. Immunol., 50, 83.

Zuckerman, A. (1977). Current status of the immunology of blood and tissue protozoa II Plasmodium. Expt. Parasitol., 42, 374-446.