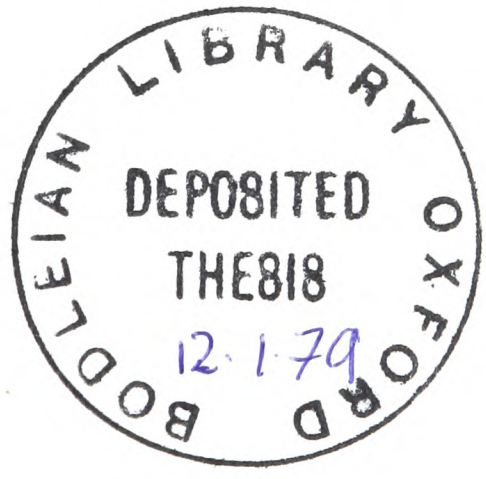
FETAL HAEMOGLOBIN AND MALARIA

by

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To my Mother and the Memory

of my Father

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ABBREVIATIONS

Kleihauer preparations

A Adult
I Intermediate
F Fetal
} cells

Parasite morphology

SR Small ring

LR Large ring

ST Small trophozoite

LT Large trophozoite

IS Immature schizont

MS Mature schizont

G Gametocyte (macro or micro)

A Abnormal form

R Ring (small and large)

T Trophozoite (small and large)

S Schizont (immature and mature)

Data presentation

D Difference (MGS of test culture - MGS of control culture)

MGS Mean growth score $\left(\frac{\text{sum of growth scores of n parasites}}{n}\right)$

R Ratio $\left(\frac{\text{rate of invasion in test cells}}{\text{rate of invasion in control cells}}\right)$

R incorp Ratio of incorporation

$$\left(\frac{\text{rate of incorporation in test cells}}{\text{rate of incorporation in control cells}}\right)$$

T/B $\left(\frac{\text{Measurement in the top fraction of a centrifuged sample}}{\text{Measurement in the bottom fraction of a centrifuged sample}}\right)$

Others

Ae Aerobic

An Anaerobic

G6PD Glucose-6-phosphate dehydrogenase

Hb Haemoglobin

Low O_2 Low oxygen tension

PBS Phosphate buffered saline

ABSTRACT

The inherited disorders of human haemoglobin are very common in some populations. Relative protection afforded to heterozygous carriers of these conditions against P. falciparum malaria is a likely cause for these high gene frequencies. However, very little is known at the cellular level of how this protective effect works. As an approach to this problem, an in vitro culture technique was developed in order to examine the factors which might modify the interaction of P. falciparum with human red cells. Fetal haemoglobin was of particular interest as a means of protection, since young infants, who are known to have high levels of this haemoglobin, are relatively resistant to P. falciparum. Furthermore, there is increasing evidence that infants heterozygous for thalassaemia and other haemoglobinopathies have higher levels of fetal haemoglobin than expected during the first year of life.

A paucity of parasites was observed in the fetal haemoglobin-containing cells in young infants but this was shown to be due to the predilection of the parasite for metabolically young cells. Fetal haemoglobin was shown to have no effect on the rates of invasion of parasites into cells from a variety of conditions. However fetal haemoglobin caused significant retardation of growth once the parasite had entered the cell. This effect was independent of cell age.

Further findings indicated that in the sickling disorders and glucose-6-phosphate dehydrogenase deficiency the red cell provided an unfavourable environment for the development of P. falciparum. This was not the case in thalassaemia and haemoglobin C and E traits. It was therefore suggested that these latter disorders have maintained their high frequencies due to the relatively high level of fetal haemoglobin which occurs in heterozygous carriers at an early stage in life, and protects them at an age when mortality from falciparum malaria is at its highest.

CHAPTER 1

GENERAL INTRODUCTION

Many of the inherited diseases affecting man are rare, and although they offer clues to the understanding of biological mechanisms, they cannot be regarded on a numerical basis, as world health problems. However amongst these, the inherited diseases affecting the red blood cell in addition to giving unique insights into inheritance and molecular disease are exceptional since they affect substantial numbers of people.

THE HAEMOGLOBINOPATHIES - DISTRIBUTION, INCIDENCE AND IMPORTANCE

Some of the inherited disorders of human haemoglobin, particularly β thalassaemia and sickle cell anaemia occur at extremely high frequencies in various parts of the world As reviewed by (Neel, 1957; Livingstone, 1967, 1971; Lehmann and Huntsman, 1974). The geographical distribution of some of these is illustrated in Figure 1. These disorders occur mainly in restricted areas of the Old World from where they have spread by migration and admixture. Thalassaemia, the most widespread of these haemoglobinopathies is found especially around the Mediterranean Sea, across the Indian subcontinent and extends into Southeast Asia (Chernoff, 1959; Weatherall and Clegg, 1972) It is generally rare in Subsaharan Africa except for areas of

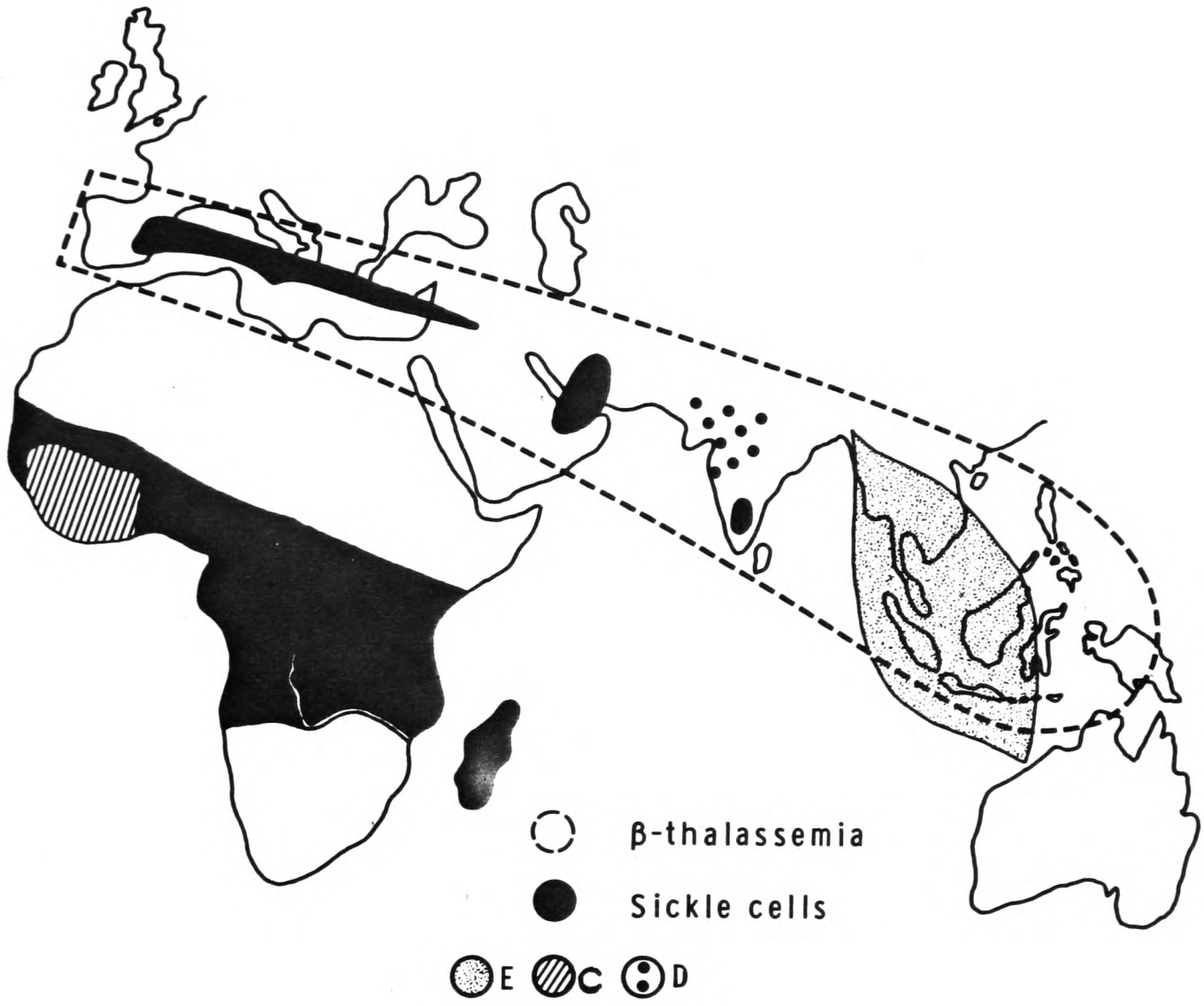


Figure 1 World distribution of the major haemoglobin abnormalities.

Northern Liberia and Ghana where it occurs in significant numbers (Weatherall et al, 1971). A population survey in Northern Liberia showed an average incidence of 9% for β thalassaemia trait, which is the highest yet recorded for an African Negro population (Willcox, 1975). However, the most commonly encountered haemoglobinopathy in these areas is sickle cell anaemia, its distribution including the belt of tropical Africa as far south as the Zambesi River (Allison, 1965; Livingstone, 1967). Sickling disorders are also found outside Africa in the broad areas outlined above where β thalassaemia is common, but it appears that the gene frequencies of these disorders are mutually exclusive (Barnicot et al, 1963; Motulsky, 1964). That is to say that in specific areas where β thalassaemia is common, sickling disorders are relatively rare and vice versa. This is presumably due to the loss of double heterozygotes, so that proportionately there is a greater loss of the less frequently occurring gene. Pockets of the sickle gene have been found in Greece (Deliyannis and Tavlarakis, 1955), Saudi Arabia (Lehmann et al, 1963; Gelpi, 1967), and in India (Chatterjea, 1966). The other common haemoglobinopathies such as haemoglobin C, E and D Punjab tend to be highly localised in areas such as Northern Ghana (Edington and Lehmann, 1954), Southeast Asia (Flatz et al, 1965) and India (Sukumaran, 1975).respectively. One further inherited disorder of the red cell that deserves mention because it is so common is glucose-6-phosphate dehydrogenase (G6PD) deficiency. Unlike the sickling disorders and thalassaemia which seldom occur together, G6PD is found both in the geographical areas and sometimes in the same populations where the sickling disorders and thalassaemia are common (Motulsky, 1964).

In addition to their widespread distribution, the incidence of these diseases can be extremely high within certain areas. For example carriers of the β thalassaemia gene can include as many as 40% of the population in the Ferrara region of Northern Italy, on the island of Sardinia and on Rhodes Island, Greece (Weatherall and Clegg, 1972). As reviewed by On Cyprus the incidence is nearer 15% (Ashiotis et al, 1973), whilst in Northern Thailand the incidence of α thalassaemia is 30% and the frequency of β thalassaemia ranges from 3-9% (Wasi et al, 1969; Wasi, 1978). For the sickle gene a similar figure of up to 40% of the population being heterozygous has been described for both West and East Africa (Allison, 1954a; Edington, 1955). G6PD deficiency is probably the most common of all inherited red cell disorders and is said to be present in 100 million individuals worldwide (Wld. Hlth.Org. 1967). In certain areas of Saudi Arabia for example, frequencies of up to 43% for deficient males have been found (Gelpi, 1965; Pembrey and Fletcher, personal communication).

The high incidence of heterozygotes for these conditions results in large numbers of homozygotes which at least in the areas of β thalassaemia and the sickling disorders, pose a major health problem because of the severity of their illness. Without constant medical attention and favourable socio-economic conditions very few homozygotes reach adult life. In Africa for example, the fitness of the sickle cell homozygote is close to zero (Allison, 1965); that is to say very few reach reproductive age. Moreover they occur most frequently in those countries which can least afford to finance such comprehensive care without detrimental effect

on the minimal resources needed for countless other health problems.

Occurring at incidences close to 40% in certain areas, the heterozygotes for these haemoglobinopathies and the homozygotes to which they give rise, pose a complex medical problem. As a comparison the next most commonly known inherited disease on a world basis is cystic fibrosis, with an incidence of heterozygotes in the Caucasian population of about 4% (Lobeck et al, 1972). Very few of the homozygotes in cystic fibrosis survive to reproduce.

POLYMORPHISM, BALANCED POLYMORPHISMS AND THE MALARIA HYPOTHESIS

The occurrence of these red cell disorders at such high frequences in certain populations constitutes the phenomenon usually referred to as a genetic polymorphism. This has been defined as "The occurrence together in the same habitat, at the same time, of two or more distinct forms of a species in such proportions that the rarest of them cannot be maintained merely by recurrent mutation" (Ford, 1940). As a general rule a polymorphism exists wherever a second genetic variant exists with a frequency of one percent or more.

Mutation seems an unlikely mechanism for the maintenance of the polymorphism of the abnormal haemoglobins, thalassaemias and G6PD deficiency. In a careful study involving 233 mothers of one or more children with sickle cell disease, 231 were found to be positive and two negative for the sickle cell trait as tested by electrophoresis (Vandepitte et al, 1955). Of the fathers, 190 were positive, 16 were negative. Of these 16, paternity was excluded in 10. They calculated on this basis that the upper limits of mutation was

1.7×10^{-3} per generation which is only about a tenth of the rate necessary to maintain the sickle gene at its known frequency. The Lambotte-Legrands (1951) had found similar figures in a study of 297 mothers of children with sickle cell disease. Allison (1956) for example has calculated on a mathematical basis that to maintain the high frequency of the sickle cell gene the mutation rate would have to be about 3000 times greater than the naturally occurring mutation rates calculated for man. Thus, if not maintained by mutation, how can these conditions, which are commonly lethal in the homozygous state with concomitant loss of genes in each generation, attain their high frequencies?

One possibility is that of balanced polymorphism, whereby individuals who are carriers for these diseases contribute on average more offspring to the next generation than either normal individuals or affected homozygotes. This occurs when they are exposed to a selective factor in the environment which carries a high risk of mortality or reduced fertility in normal individuals, but from which heterozygotes are relatively protected. Thus, such carriers increase in numbers in successive generations as long as the particular environmental conditions which favour them are present. However a situation of equilibrium is reached when the increase in heterozygotes is balanced by the loss of increasing resultant homozygotes. Thus the mutant haemoglobin does not ever totally replace normal haemoglobin A (Hb A).

It was J.B.S. Haldane in 1949 who first suggested what this selective factor might be in the case of the

the haemoglobinopathies. He postulated that infectious diseases in general had been important evolutionary agents and that in this connection malaria caused by Plasmodium falciparum was no exception. This disease has a high mortality and even in present times is responsible for over one million deaths per year in Africa alone (Wld. Hlth.Org. 1975). Young children are most often involved (Garnham, 1949; Bruce Chwatt, 1952; Colbourne and Edington, 1954). There is also evidence of decreased fertility of mothers with malaria resulting in abortions, premature labour and stillbirths (MacGregor and Avery, 1974). Moreover there is a remarkable coincidence in the geographical areas which are (or have been) endemic for malaria and those in which the haemoglobinopathies are common (Fig. 2). Even around the Mediterranean basin where thalassaemia is common, falciparum malaria was only finally eradicated after the Second World War. Malaria seems a likely selective factor in the case of red cell disorders since it is the infection of the red cell itself by the parasite which is responsible for the clinical signs and complications of the disease and which determines the fatal or non-fatal outcome.

Haldane (1949) originally hypothesised that heterozygotes for β thalassaemia might be more resistant to falciparum malaria because the red cells in this condition are smaller and more resistant to hypotonic lysis. He suggested that genetic equilibrium would be reached when the loss of thalassaemia genes due to premature death of homozygotes was balanced by a corresponding loss of normal genes due to the increased susceptibility of normal individuals (compared with thalassaemia heterozygotes) to fatal infection with



Figure 2 Distribution of malaria in areas of the world where the haemoglobinopathies are common. (Compare with Fig. 1)

falciparum malaria. This hypothesis has now been extended to explain the high incidence of many of the other inherited disorders of red cells. Where malaria has become implicated as the selective factor, this argument is called the malaria hypothesis.

EPIDEMIOLOGICAL EVIDENCE TO SUPPORT THE MALARIA HYPOTHESIS

Most of the data supporting the malaria hypothesis are of an epidemiological nature and although the hypothesis includes thalassaemia and other inherited red cell disorders, most of the data havebeen concerned with sickle cell anaemia.

Sickle cell anaemia

Beet (1946 and 1947) working in Northern Rhodesia (present-day Zambia) was the first to report that the malaria parasite rate (the percentage of the population infected) was lower in carriers of the sickle cell trait than in those without the trait even though these differences were not statistically significant. In his second study he also found that trait carriers less frequently had enlarged spleens, but attributed this to repeated infarction with resultant shrinkage of the spleen rather than to less severe malaria. Brain (1952a) confirmed the latter finding but suggested that this lower incidence of splenomegaly might reflect a diminished susceptibility to malaria on the part of the sickle carriers. Indeed further work (Brain, 1952b) demonstrated that malaria seemed to be less common in a small group of trait carriers than in another group without the trait although his results were not statistically significant.

Much of the epidemiological data to support the malaria hypothesis comes from Allison (1954). He found that amongst schoolchildren in Kampala, Uganda, 43 with a positive metabisulphite test for sickling had a lower parasite rate (27.9%) for P. falciparum than a group of 247 children without sickle cells (parasite rate 45.7%). The parasite count (percentage of red cells infected) was also significantly higher in non-sicklers. In another study reported in the same paper 30 adult male Africans, who had been absent from a malarious area for at least 18 months were infected with a Malayan or East African strain of P. falciparum (Allison, 1954). Fifteen were sickle cell heterozygotes whilst fifteen were non-sicklers. All but one individual without the sickle cell trait developed parasitaemia whereas only two of the sicklers showed the presence of parasites, and in them the parasite counts were low. Beutler et al (1955) attempted a similar experiment, but used a Panamanian strain of P. falciparum to inoculate 16 volunteers, eight sickle cell heterozygotes and eight normals, who had previously not been exposed to malaria. To determine the effect of acquired immunity on the resistance of sicklers and non-sicklers they reinfected five in each group after a lapse of four months. In both cases they were able "to demonstrate only unimpressive differences of questionable significance." Raper (1955), studying 200 children under the age of 10 years, was able to show that P. falciparum was noticeably commoner in non-sicklers, except in the first year of life. Moreover the average parasite density in sicklers was lower than that of non-sicklers in this group. However, further groups of 1,194 children and 663 adults showed no significant

difference in parasite rate between sicklers and non-sicklers. Raper proposed that the presence of Hb S did not prevent infection, but limited the extent to which a falciparum infection could develop. In a further study, the same author (Raper, 1956) showed that of 13 patients with sickle cell trait who were in hospital with malaria, none had cerebral malaria or blackwater fever whereas in 123 non-sicklers with malaria, 47 had cerebral malaria and six blackwater fever. None of the sicklers died whereas there were 16 deaths attributable to malaria amongst the non-sicklers.

Further epidemiological data on the malaria hypothesis in sickle cell anaemia abounds, and has been extensively reviewed (Neel, 1956; Allison, 1957, 1961, 1964, 1965; Motulsky, 1964; Luzzatto, 1972, 1974, 1975: Livingstone, 1971). The sort of data supporting this hypothesis include:

1) The coincidence of the geographical distribution of falciparum malaria and sickle cell anaemia (Livingstone, 1967).

2) Micromapping of malaria and sickle cell anaemia. This is a term used to describe a correlation between malaria endemicity and the frequency of polymorphic genes within a restricted geographical area (Livingstone, 1971; Siniscalco et al, 1966).

3) Age stratification. This describes the finding of an increasing percentage of sickle cell trait carriers amongst groups of individuals of increasing age in the same population (Allison, 1956; Ringelhann et al, 1976). This implies that proportionately more sickle carriers are surviving to adulthood than normals. It also argues against the suggestion of increased fertility alone being

the cause of the high frequency of heterozygotes seen. If that were the case, the percentage heterozygotes amongst various age groups would be similar.

4) An increase in live offspring born to sickle cell trait mothers living in malarious areas (Stamatoyannopoulos et al, 1973).

5) A decrease in the average spleen index (a measurement of splenic enlargement) in sickle carriers (Boyo, 1972).

6) A lower parasite rate and count as mentioned before (Allison, 1954; Raper, 1955; Colbourne and Edington, 1956; Neel, 1957).

7) Finally, and probably most important of all from a selective survival point of view, a decreased mortality amongst sickle carriers due to falciparum malaria (The Lambotte-Legrands, 1951; Raper, 1956; Vandepitte and Delaisse, 1957).

Initially the data which did not support the malaria hypothesis for sickle cell anaemia were sizeable (Moore et al, 1954; Edington, 1954; Foy et al, 1956; Archibald and Bruce Chwatt, 1955). As Neel said (1956) "From the very lively exchange of viewpoints which has characterised these publications it has become apparent that no one's series is quite comparable with anyone else's, each series having its strengths and weaknesses. Thus Foy et al (1956) criticise with some justification Raper's (1955) use of outpatient material and Raper criticises, again with some apparent justification, the failure of Moore et al (1954) to stratify their sample by age. All investigators seem united in a healthy respect for the complexity of the problem!" However, from these early studies have arisen three important considerations with regard to gathering such epidemiological

data:

1) Of the four species of plasmodia involved in causing malaria in humans, evidence for protection against P. falciparum malaria should be pursued especially since it has a strikingly higher mortality rate than malaria caused by the other species (Wilcocks and Manson Bahr, 1972).

2) Young children from about six months to two years are the group most at risk of malaria since it is approximately at this age that humoral immunity is at its lowest (McGregor, 1974). Thus it is also at this age that mortality from malaria is at its highest (Bruce Chwatt, 1952). In adult life acquired immunity would obscure any protective effect due to intrinsic properties of the red cell.

3) Mortality from falciparum malaria is the important criterion in assessing protection rather than the parasite rate. Failing this, the severity of the infection as gauged by the parasite count is of value as there is a relationship between parasite density and mortality (Field, 1949). Thus the protection of heterozygotes for the red cell disorders need only be relative and does not exclude their being infected.

It seems that, if these factors are taken into account, the malaria hypothesis holds firm for the sickle cell trait. Although investigated to a lesser extent, factors other than malaria have been implicated in maintaining the high frequencies of the sickle gene. Serjeant et al (1968) have shown that in more recent times, with increase in living standards, homozygous sicklers can live long enough and reproduce. Roberts and Boyo (1960) have suggested that sickle cell heterozygotes, male and female, have enhanced fertility. This would occur in the absence of malaria.

Finally, since polygamy is so widely practised in Africa, it has been mathematically postulated that were male sickle cell trait carriers, who are polygamous, to have on average more children, the frequency of the sickle gene would rise in the population (Konotey Ahulu, 1970; Bonney and Konotey Ahulu, 1977). There have however been no data documented to support this theory.

Glucose-6-phosphate dehydrogenase (G6PD) deficiency

Second to sickle cell anaemia, much of the literature dealing with the malaria hypothesis has centered on G6PD deficiency. This is the most common defect of red cell metabolism known, with more than 100 million individuals affected worldwide (Wld.Hlth.Org. 1967). Motulsky (1960) was first to emphasise the geographic coincidence of the disease and falciparum malaria. He proposed that as in sickle cell anaemia, the malaria hypothesis could explain the high frequencies of this inherited sex-linked disorder. Siniscalco et al (1966) presented unique epidemiological data on the island of Sardinia which showed a decrease in the incidence of both G6PD deficiency and thalassaemia with an increase in altitude which corresponds with the prevalence of malaria in the past. This was heaviest at the coast and minimal in the mountainous regions. This, he felt, suggested that malaria might well have been a strong selective factor in the past favouring heterozygotes for these diseases. However, comparison of parasite rates and counts amongst normal and deficient individuals have been contradictory. Thus Allison and Clyde (1961), Harris and Gilles (1961), and Gilles et al (1967) found lower parasite counts in young enzyme-deficient children compared to normals,

whilst Kruatrachue et al (1962, 1966, 1970), Motulsky (1965), Edington and Watson-Williams (1965) and Segal et al (1972) found no such difference. But the last-mentioned study for example involved persons of all ages and therefore acquired immunity could have possibly invalidated the data. Finally, Powell and Brewer (1965) could find no difference between eight individuals deficient in G6PD and eight normals, when inoculated with P. falciparum with mosquito-induced infection. This was both in terms of the prepatent period (time from inoculation to the appearance of parasites in the blood) and in parasite counts.

These contradictory results are not entirely unexpected because it has subsequently been found that G6PD deficiency is a heterogeneous disease (Wld.Hlth.Org. 1967) since many variants of the enzyme have been found to exist, each with relatively different activity from the normal enzyme (B variant) Some structurally differing variants in fact show normal activity e.g. the A variant occurring commonly amongst African populations. There is some evidence that even this non-deficient variant might protect against falciparum malaria (Bienzle et al, 1972). Thus relative resistance may not be a simple function of the G6PD activity in red blood cells.

Haemoglobins C and E

Epidemiological evidence for the malaria hypothesis in carriers for Hbs C and E is less convincing than that for sickle cell trait or G6PD deficiency. Thus although Edington and Watson-Williams (1965) found only two deaths due to malaria in Hb C carriers where 4.5 were expected, and Thompson (1962, 1963) found lower parasite counts in

Hb C carriers particularly when high densities of parasites were compared, other workers have found no differences either in parasite rates or counts when normals and Hb C carriers were compared (Edington and Laing, 1957). In a study involving Hb E heterozygotes, Kruatrachue et al (1969) could find no evidence of decreased parasite rates or counts amongst such individuals to support the malaria hypothesis, and this same group (Kruatrachue et al, 1970) was unable to find any difference in the frequency of cerebral complications of malaria in Hb E carriers and normals. The broad geographical correlation of falciparum malaria and Hb E nevertheless remains very suggestive. For example in Northeast Thailand, where malaria is common, the prevalence of heterozygotes for Hb E reaches a remarkable 53% (Na-Nakorn and Wasi, 1978). However as Livingstone (1971) correctly points out, the relative fitness of Hbs C and E homozygotes is far higher than that of Hb S (three times as an estimate) so that the advantage for heterozygotes need only be slight to maintain the high frequencies of Hbs C and E.

Thalassaemia

Evidence that protection of heterozygotes against malaria might have maintained the thalassaemia polymorphism has been extensively reviewed by Chernoff (1959), Bannerman (1961), Motulsky (1964) and Weatherall and Clegg (1972). The overall picture is again one of a close geographical correlation of areas which are or have been endemic for falciparum malaria and where thalassaemia is common. The data of Siniscalco et al (1966) mentioned above in relation to malaria endemicity and G6PD deficiency found the same correlation in the island of Sardinia where the prevalence

of thalassaemia fell with a rise in altitude. This correlates well with past malaria which was most prevalent at the coast and almost absent in the mountainous regions. However, for thalassaemia there are no data on parasite rates and densities in the blood or mortality studies which have been central to testing the malaria hypothesis for sickle cell trait and G6PD deficiency. It should also be added that some studies have shown no correlation of thalassaemia and malaria endemicity in Greece (Stamatoyannopoulos and Fessas, 1964) and on Cyprus (Plato et al, 1964).

EVIDENCE AT THE CELLULAR LEVEL TO SUPPORT THE MALARIA HYPOTHESIS

Many of the epidemiological data are difficult to interpret because of uncertainties concerning the selection of suitable and representative population samples. Further problems as mentioned earlier include the need to specify the species of malaria studied, the age group of individuals concerned and whether the mortality observed in such cases can be directly attributed to malaria, particularly in those geographical areas where multiple pathologies are so common. It is therefore at the cellular level that data in support of the malaria hypothesis are most needed. However, none of the past studies of these haemoglobinopathies have satisfactorily demonstrated how the interaction of parasite and red cells from heterozygotes for these conditions might be responsible for the protection afforded against malaria. Such evidence would strongly support the malaria hypothesis. To understand how protection is provided at the cellular level it is first necessary to examine the interaction of the parasite and the normal red cell fully to appreciate how the

abnormal red cell might alter the outcome of an infection.

Invasion and growth of the malarial parasite in normal red cells

The relationship of the malaria parasite and the red cell is a particularly intimate one. At the onset of the asexual cycle, merozoites[1] released from hepatocytes in the liver invade uninfected red cells and then following growth and division give rise to a generation of erythrocytic merozoites which cause the red cell to rupture and invade further uninfected cells. This cycle repeated many times allows for a rapid multiplication of parasites (Fig. 3).

The process of red cell invasion by a parasite is believed to follow a well ordered sequence of specific events (Miller, 1977). This remarkable process has been filmed by means of an electro-optical system which can record microscopic images at low light intensities (Dvorak et al, 1975). In schizonts of P. knowlesi in rhesus monkey erythrocytes, the first change prior to the release of merozoites is the coalescence of the malaria pigment which has accumulated due to the breakdown of haemoglobin and consists mainly of iron-containing haem, into a single unit within a residual body. In P. falciparum by contrast the pigment is at this stage already contained in a single clump. Small vesicles then develop on the red cell membrane surface which becomes distorted, presumably due to movement of the

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1 Greek derivation meros - part + zoon - animal

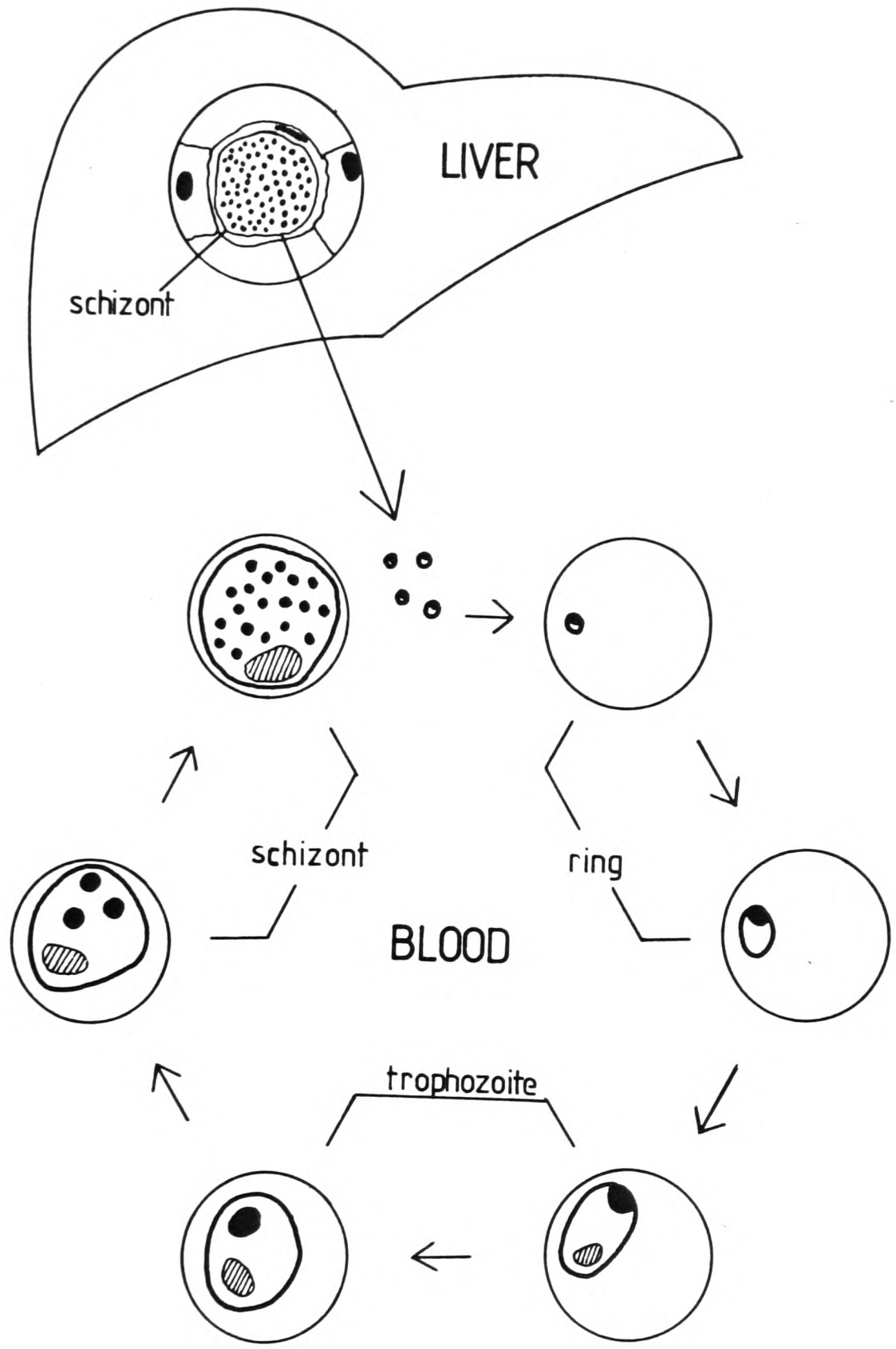


Figure 3 The asexual erythrocytic cycle of P. falciparum

intracellular merozoites. Swelling is finally followed by explosive rupture of the infected cell. The time taken from formation of these vesicles to when the cell ruptures is of the order of about a minute. Each merozoite measures about 1x1.5 μ. The released merozoites for which no evidence of motility has yet been demonstrated, then make contact with an uninfected cell, but for penetration to occur the paired organelle at the apical region of the merozoite must attach itself to the red cell membrane (Miller, 1977). It has been suggested that there is a specific receptor site on the red cell membrane where this occurs (Miller et al, 1973). The red cell is then deformed, losing its biconcave shape for up to 10 seconds but then returns to its normal shape with the merozoite still attached at its initial point of contact. This is followed by a process of interiorisation resembling endocytosis whereby the parasite comes to lie within the red cell, but is still enclosed by a membrane of red cell origin (Fig. 4). This process takes about 10 to 20 seconds and is followed by a further wave of deformation on the red cell surface. Thereafter the orifice of the vacuole in which the parasite is contained is sealed. Lysis may occur spontaneously upon invasion, and always follows simultaneous invasion by two or more merozoites of P. knowlesi or if the merozoite is still attached to the pigment-containing residual body. The common observation of multiply infected cells in falciparum malaria however would indicate that this does not hold in infections by this species or that alternatively human red cells are capable of sustaining multiple infections. It should also be emphasised at this stage that although the parasite comes to lie inside the red cell, it is still

effectively extracellular as it is surrounded by a membrane that is derived from the red cell surface (Fig. 4). This has been confirmed by electron microscopic studies (Ladda et al, 1969) which show that the intraerythrocytic parasites in P. berghei and P. gallinaceum infections have both a limiting membrane of parasitic and one of erythrocytic origin around them.

Parasite growth occurs primarily by metabolising the haemoglobin of the host cell (Sherman, 1977) by a process of phagotrophy, a form of pinocytosis described by Rudzinska and Trager initially for P. lophurae (1957) but later found to be similar for P. falciparum (Rudzinska et al, 1960). Sherman (1977) has postulated the following mechanism by which the parasite metabolises haemoglobin:- the two membranes separating the parasite from the host cytoplasm invaginate as the haemoglobin is ingested (Fig. 4). Small haemoglobin-containing food vacuoles are pinched off and proteolysis of the globin moiety occurs after the haemoglobin has been first auto-oxidised to methaemoglobin and non-enzymically dissociated into haematin and globin. The haematin thus formed accumulates in these food vacuoles as pigment granules which coalesce to form haemazoin (Moulder, 1962). The proteolysis of the globin moiety is thought to occur either as a result of the membrane of the food vacuole taking on lysosomal characteristics, or by means of proteases secreted from the parasite cytoplasm into the vacuole (Aikawa and Thompson, 1971).

Morphological changes take place in the parasite during this growth period (Plate VI a-h). The small ring forms, about 1.5 μ in diameter and each with a nucleus and

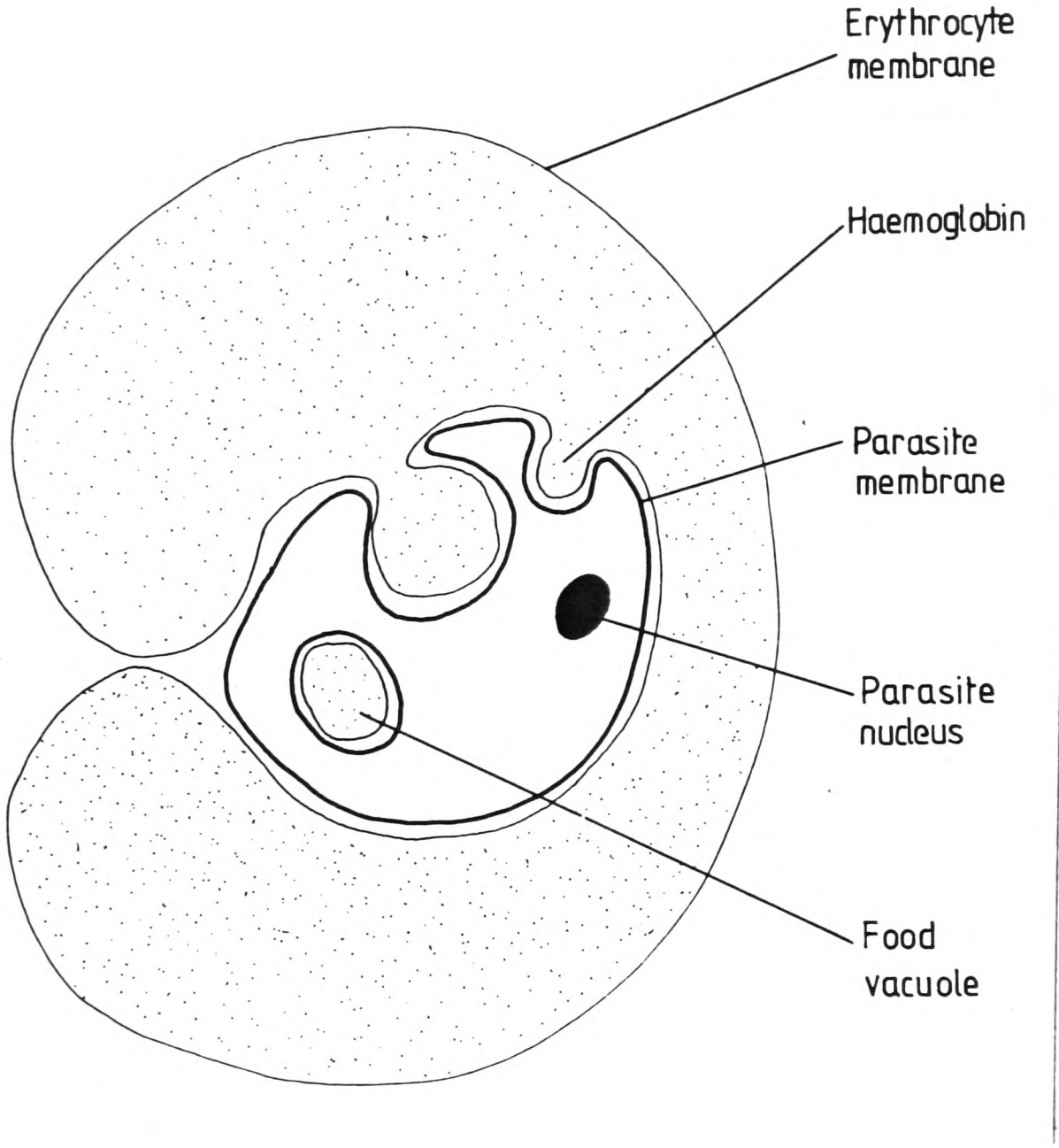


Figure 4 Diagrammatic representation of the relationship of the red cell and parasite membranes of a parasite metabolising haemoglobin.

cytoplasm surrounding a large vacuole, first enlarge giving rise to large rings. Later the accumulating single clump of haemazoin (the residual body) becomes visible and at this stage the parasite is called a small trophozoite[1] which then grows into a large trophozoite. After about 36 h of the 48 h asexual cycle (Siddiqui and Schnell, 1972), the nucleus subdivides to form the immature schizont[2] which matures and ultimately has 16 - 32 nuclei, each surrounded by a portion of cytoplasm. The mature schizont now occupies the greater part of the red cell and has metabolised most of the haemoglobin inside the cell. In P. knowlesi for example the parasite utilises approximately 75% of the haemoglobin inside a normal red cell (Moulder, 1962). Each nucleus surrounded by its portion of cytoplasm is easily visualised on staining for parasites and is known as a merozoite and it is this form of the parasite that invades uninfected cells and gives rise to the next generation. While most of these merozoites will give rise to a further asexual cycle some will develop quite differently into male and female sexual forms called gametocytes which are essential for the continuation of the life cycle of the parasite by mosquito transmission.

The mechanism of protection at a cellular level - Luzzatto's Postulates

This description of the normal erythrocytic cycle of the parasite should partly clarify how variations in the red cell, particularly with regard to haemoglobin content,

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1 Greek derivation trophē - food

2 Greek derivation skhizō - to split

might alter the outcome of the infection of such cells by parasites. Luzzatto (1975) has proposed on a theoretical basis, three mechanisms by which a red cell might be unfavourable to a parasite (Fig. 5).

1) The parasite might be unable to invade the red cell, "failed infection".

2) The parasite might invade the red cell but then fail to develop inside it, "abortive infection".

3) Having entered the red cell, the parasite might grow to some extent but in so doing cause premature removal of such a cell from the circulation, "suicidal infection".

Evidence for the existence of these three mechanisms of protection in certain of the haemoglobinopathies is discussed below.

Sickle cell anaemia

Numerous cellular mechanisms for protection against malaria have been suggested for sickle cell anaemia and in this sense it has served as the prototype for the other haemoglobinopathies since it is the most prevalent and best defined. When Allison wrote his paper on the protection of sickle cell heterozygotes in falciparum malaria (1954), he postulated that sickle cells were less easily invaded than normal erythrocytes because of their abnormal haemoglobin content. Alternatively he suggested that although the parasite was capable of metabolising the haemoglobin almost completely in the normal intact red cell, this might not be the case with relatively insoluble sickle haemoglobin. He did however emphasise that this was a relative effect,

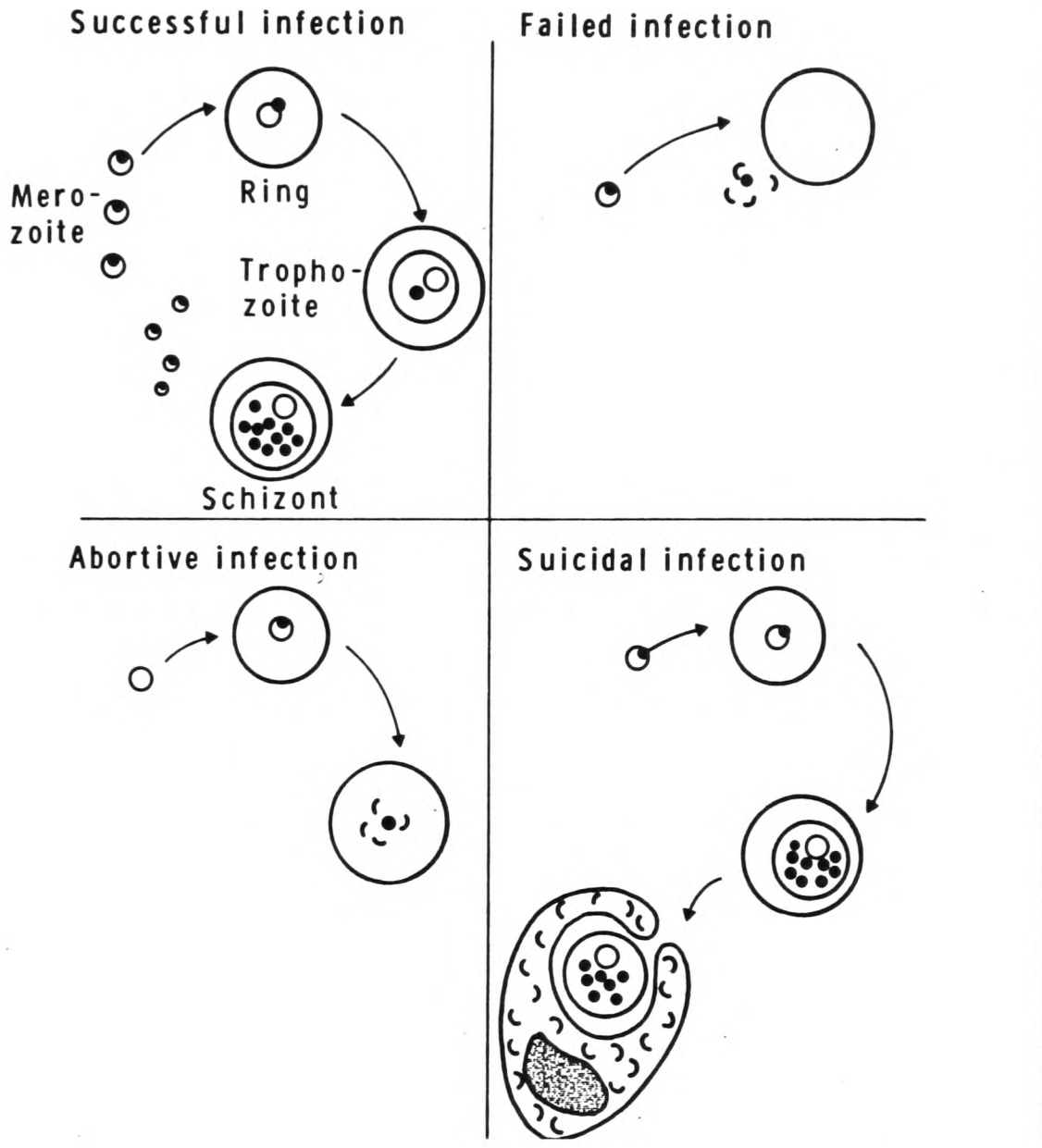


Figure 5 Diagrammatic representation of three mechanisms by which the red cell might be disadvantageous to a malarial parasite (from Luzzatto, 1975).

a fact borne out by Raper (1955) who noted that even in the red cells from homozygote sicklers which contain predominantly Hb S with small amounts of Hb F and Hb A_2 but no Hb A, parasites were able to exist, mature and even multiply.

Many of the data which form the basis for an explanation of the mechanism of protection in sickle cell anaemia rely on comparison of the distribution of parasites between sickled and non-sickled cells in infected blood after the addition of sodium metabisulphite, a strong reducing agent which induces sickling of cells containing Hb S. Mackey and Vivarelli (1954) found in stained thin films that there was a paucity of parasites in the cells that had sickled and they interpreted this as a failure of the parasites to invade such cells. Miller et al (1956) on the other hand, found an equal distribution of parasites between sickled and non-sickled cells in two cases and only in a third case was there a paucity of parasites in the sickled cells. Contrary to Mackey and Vivarelli (1954) they felt this indicated that the parasites were able to invade and develop in the early stage at least in sickled cells. However they did state that if Hb S were distributed amongst all the cells of such sickle heterozygotes, which has subsequently been found to be so (Papayannopoulou et al, 1976) then the possibility remained that sickle heterozygote cells as a whole could be less parasitised than cells from an individual with normal adult haemoglobin. What they did not specify were the conditions under which this would occur.

The first attempt at culture of parasites in cells from a sickle heterozygote was undertaken by Raper (1959). Under culture conditions of both 6% and 1% oxygen in cultures which were shaken he was unable to show any difference in growth between those parasites in cells containing Hb S and those grown in normal adult red cells. This, he felt, implied that the parasite was capable of metabolising Hb S. However very little sickling was evident in his cultures for, even when exposed to 1% oxygen continuously for 27 h, only 8% of the cells had sickled.

The emphasis thus moved away from the actual metabolism of the Hb S by the parasite since it seemed quite capable of growing in cells containing Hb S and more interest was placed on the actual event of sickling. Miller (1956) suggested that when such cells infected with the more mature forms of P. falciparum became lodged in the deep tissues they were unable to replenish their oxygen supply and would thus be induced to sickle, causing death of the parasite. Allison (1957) pointed out however that the oxygen tension in these deep tissues was insufficient to cause sickling of erythrocytes from sickle cell heterozygotes. Thus, whereas the oxygen tension in mixed venous blood is of the order of 45 mmHg, present data (Milner, 1974) indicates that oxygen tensions of at least 25 mmHg are required for sickling of cells from a sickle heterozygote to commence. Thus it seemed unlikely that the oxygen tension in the deep tissues was sufficiently low for red cells from a Hb S heterozygote to sickle. Luzzatto et al (1970) were able to circumvent this problem by proposing that the parasite itself vigorously utilized oxygen and could thus reduce the oxygen tension

enough to induce sickling. They found that, under defined experimental conditions in cultures of P. falciparum-containing cells from sickle heterozygotes and in the presence of 8% metabisulphite, there were more parasites in the sickled cells, the opposite of the results obtained by Mackey and Vivarelli (1954) and in one of the cases of Miller (1956). Furthermore the rate of sickling under these conditions was significantly higher in the parasitised than in the non-parasitised cells. Luzzatto suggested that these findings supported his third mechanism of protection of red cells against P. falciparum malaria, namely suicidal infection; and this remains the currently favoured theory. He explained it as follows:- The parasite enters a red cell containing Hb S and in its development utilises oxygen sufficient to cause the cell to sickle. This sickled parasitised cell is, because of its shape, removed from the circulation before the parasite can develop further.

Certain criticisms may be made of this paper. Firstly, as his cultures contained 8% metabisulphite and were carried out under paraffin oil, they are highly unphysiological. The presence of such a powerful reducing agent probably exceeds any reduction in oxygen tension found in vivo even in the deep tissues, and may itself be toxic to the parasite. It may be questioned whether in fact the parasite could grow at all under such conditions. If parasitisation in itself were sufficient to drop the oxygen tension in vivo as he proposed, then culture under paraffin oil alone without metabisulphite should have been sufficient to demonstrate this effect. This of course

presumes that the parasite uses oxygen, which is still uncertain (Homewood, 1977). Even if the parasite does require oxygen it seems puzzling that diffusion of oxygen from neighbouring uninfected red cells should not occur and prevent sickling of those cells containing parasites. One further reservation is that although it has been shown that both sickled cells (Bunn et al, 1977) and parasitised cells (Conrad, 1969) have a shortened lifespan, there is no evidence to suggest that sickled parasitised cells are removed in preference to unsickled parasitised cells. In any event, Luzzatto et al (1970) showed that on exposure to oxygen nearly all the sickled cells, infected and uninfected, unsickle almost simultaneously. Thus, after entering the peripheral circulation most infected cells would rapidly unsickle and remain so unless trapped in the deep tissues for prolonged periods. Furthermore their experiments were not performed on the mature forms which are those found in vivo in the deep tissues.

The data on the possible cellular mechanisms of protection have been presented to show that it is still unclear exactly how this occurs in sickle cell heterozygotes and that the currently favoured mechanism rests on unconfirmed assumptions. Other mechanisms not involving the interaction of the red cell and the parasite have been suggested for sickle cell anaemia. Edozien et al (1960) showed that sickle carriers have significantly higher levels of γ globulin than non-sicklers. That this was most likely due to malaria was shown by a control group of sickle carriers and non-sicklers given weekly antimalarial prophylaxis. In this case the difference between γ globulins

in sicklers and non-sicklers was hardly evident. However since much of the antibody produced during an infection has been shown to have little protective value (Cohen and Butcher, 1970; Butcher et al, 1970) it cannot be ascertained whether this difference in γ globulins observed is of any significance in terms of actual protection afforded to sickle heterozygotes.

More recently Laser and Klein (1977) have proposed an even more ingenious protective mechanism for those individuals with Hb S. They noted that the level of serum fatty acids rose in a malarial infection and that these might be implicated in the destruction of erythrocytes if they exceed the buffering capacity of the available protein present. They demonstrated that Hb S could delay haemolysis of red cells far better than Hb A in the presence of oleic acid presumably on the basis of the increased buffering capacity of Hb S. They concluded that this delayed haemolysis might prevent or retard the release of merozoites from the red cell. On a purely hypothetical basis, this seems unlikely. One might, if anything, argue the opposite on the basis of these observations; namely that a tendency towards lysis as was demonstrated in the Hb A containing cells might protect the individual by a mechanism of suicidal infection. In such an argument it might be postulated that premature lysis of infected cells would occur before the parasite inside them had matured fully. Haemoglobin S on the other hand would sustain full parasite development.

In summary therefore it can be said that although the malaria hypothesis has been well documented for the maintenance of the sickle polymorphism, the exact mechanism by which this

occurs is not known. The theory of suicidal infection as proposed by Luzzatto et al (1970) is currently favoured, but is not entirely satisfactory. For the other red cell disorders the mechanism(s) is (are) even less clear.

G6PD deficiency

At the cellular level Luzzatto et al (1969) found that in infected female heterozygotes for G6PD deficiency, who because of random inactivation of one of the two X chromosomes in each cell (Lyonisation) have a mixture of normal and enzyme deficient cells, there was a 2 to 80 times greater probability of finding parasites in normal cells as compared to those deficient in G6PD. This was called a preference of the parasite for normal cells, the mechanism of which could have resulted from one of the three mechamisms of failed, abortive or suicidal infection.

A further paper on the mechanism of protection involved in G6PD deficiency favours the suicidal infection model (Eaton et al, 1976). These experiments were conducted on vitamin E deficient mice infected with P. berghei. These authors claimed that vitamin E deficiency confers a state of oxidant sensitivity in red cells not unlike G6PD deficiency, and that such mice were more resistant to P. berghei infections, as manifested by a slower development of parasitaemia and longer survival. Their evidence suggested that deficient and normal erythrocytes were being infected with equal frequency but that the vitamin E deficient cells were unable to support parasite development and were preferentially lysed, thus invoking the mechanism of suicidal infection.

Thalassaemia

The red cells from heterozygotes for β thalassaemia differ in many respects from normal adult cells not only with regard to small cell size and degree of haemoglobinisation, but also in changes in cell metabolism, permeability of the membrane particularly to potassium and increased resistance to osmotic lysis (Knox Macaulay and Weatherall, 1974). In these respects they are very similar to the cells seen in other forms of hypochromic anaemia. Thus there may be many factors present in such thalassaemic cells unfavourable to parasite growth. Brewer and Powell (1965) and Eaton and Brewer (1969) have suggested that the reduced level of adenosine triphosphate (ATP) found in the cells of individuals with β thalassaemia trait might be implicated in the protective mechanism against the malarial parasite. However, such lowered levels in the steady state have not been substantiated (Knox Macaulay and Weatherall, 1974). It does indeed seem strange that no cellular evidence of protection for β thalassaemia exists even though Haldane's original malaria hypothesis (1949) not only singled out thalassaemia but even specified that it was the small hypochromic cells found in this condition that might be responsible.

Haemoglobins C and E

No evidence exists at a cellular level for any protection afforded against malaria by Hbs C or E. In heterozygotes for Hb C, the red cells contain about 30 to 40 % Hb C (Bunn et al, 1977) and often appear as target cells on a stained blood smear, but have a normal lifespan (Prindle and McCurdy, 1969). Haemoglobin C is less soluble than Hb A and tends

to form crystals particularly if the red cells are suspended in hypertonic solutions. Haemoglobin E carriers on the other hand have about 30% of the abnormal haemoglobin but no haematological abnormalities have as yet been described for these heterozygotes (Bunn et al, 1977).

Whether the cells in heterozygotes for Hbs C or E have any deleterious effect on the proliferation of falciparum parasites is as yet unknown.

IS THE MALARIA HYPOTHESIS AN ADEQUATE EXPLANATION FOR THE MAINTENANCE OF THESE INHERITED RED CELL DISORDERS?

Many of the inherited disorders of red cells whose frequencies in parts of the world are higher than expected, have thus been accounted for by preferential survival in infection by P. falciparum. However the exact cellular mechanism by which this occurs is unknown and many such mechanisms may be involved, especially since these are a widely varying group of diseases with a broad spectrum of cellular defects. For many of these disorders evidence for the malaria hypothesis has not been confirmed and many questions remain unanswered. These problems include:-

1) In the sickling disorders it is most likely that it is the properties of the abnormal haemoglobin that provides some degree of protection against P. falciparum. On the other hand in the thalassaemia syndromes the cells do not contain a haemoglobin variant although they do contain substantial amounts of free α chains (Weatherall and Clegg, 1972). Even in the other common disorders due to β chain haemoglobin variants such as Hbs C or E which differ functionally only slightly from Hb A, it is not clear how

protection against P. falciparum might be mediated.

2) Both the thalassaemias and G6PD deficiency have been found to be heterogeneous diseases. This might possibly necessitate a variety of protective mechanisms, different in each case.

3) Since these red cell disorders are so common, it might be presupposed that genetic variants of malarial parasites might have arisen which are capable of existence in such cells. However, if in fact this is the case, they have not yet been reported. The malaria parasite is genetically an extremely agile organism perhaps best demonstrated by its capacity to evade host immune mechanisms by antigenic variation (Brown, 1977) and its ability to alter preference for young red cells (Walliker, 1976). It might be expected therefore that in highly endemic areas for malaria where the inherited red cells are common, the parasite might employ similar ingenuity to overcome the unfavourable host erythrocyte environment.

4) Since the homozygous conditions for Hbs C and E and G6PD deficiency are not necessarily lethal, such red cell disorders need only small differences in protective advantage to maintain their high frequency. Such slight protection might be too subtle to detect epidemiologically. It could be possible that it is the homozygotes for these conditions that are at some minor advantage. Thus these polymorphisms might not as yet have reached stable equilibrium.

TO WHAT EXTENT COULD FETAL HAEMOGLOBIN AND ITS INTERACTION WITH THE MALARIA PARASITE PROVIDE AN ANSWER TO SOME OF THESE PROBLEMS?

In an attempt to answer some of the problems outlined above it seemed reasonable to consider the role of fetal haemoglobin (Hb F) in malarial infections to see if it had any protective function. The literature on the subject had certainly suggested this possibility but actual data on the subject were minimal.

The idea that Hb F might protect in falciparum infections is not a new one. Lehmann (1953) noted that cells from the umbilical cord of newborns (cord cells) which contain this haemoglobin, as well as cells of β thalassaemia and sickle cell homozygotes and some sickle cell heterozygotes could induce a haemagglutinating antibody which did not react with normal cells (Ikin et al, 1953). Lehmann suggested that these cells with a component of their red cell envelope not found in normal adults, might in some way be deleterious to the parasite. Allison (1954) was the first to implicate Hb F itself as being responsible in the protection of newborns against malaria, since the disappearance of these cells containing such a haemoglobin was paralleled by an increasing susceptibility to malaria. Edington and Lehmann (1956) commented on Hb F and its presence in thalassaemia heterozygotes and newborns which could be of protective value against malaria. Gilles (1957) working in the Gambia confirmed the suggestion of increasing susceptibility to malaria with decreasing levels of Hb F. He concluded that the relative insusceptibility of newborns might be the

result of a number of factors, difficult to dissociate but that Hb F could play a more important role than had been previously attributed to it. If the presence of Hb F is relevant in malaria infections, it becomes important to know more of the pattern of its production, particularly with regard to its disappearance during the switch from Hb F to Hb A and how the inherited red cell disorders might modify this changeover.

During human development there are two major haemoglobins present. From about the eighth week of gestation the production of Hb F accounts for 90-95% of the total haemoglobin, the remainder being largely Hb A (reviewed by Wood et al, 1977). The proportion of Hb F falls from about 32-36 weeks gestation so that at birth it accounts for about 70-90% of the total (Fig. 6). It continues to decline during subsequent months to reach adult levels of under 2% by 6-12 months. However, recent data have shown that in heterozygotes for sickle cell anaemia and β thalassaemia, Hb F persists after birth at higher levels and for a longer period than in normal infants (Beaven et al, 1961, Alter, 1978, Serjeant, personal communication). Beaven et al (1961) found that both in thalassaemia minor and sickle cell trait (three cases of each) the levels of Hb F remained elevated, even at 9-12 months. The percentage Hb F extrapolated for 6-month-old infants would be of the order of 10-20% of total haemoglobin. Even more recently Alter (1978) showed that five individuals with β thalassaemia trait had significantly raised levels of Hb F, the most marked being 10% at 14 months. Finally, it should be added that a certain percentage of β thalassaemia

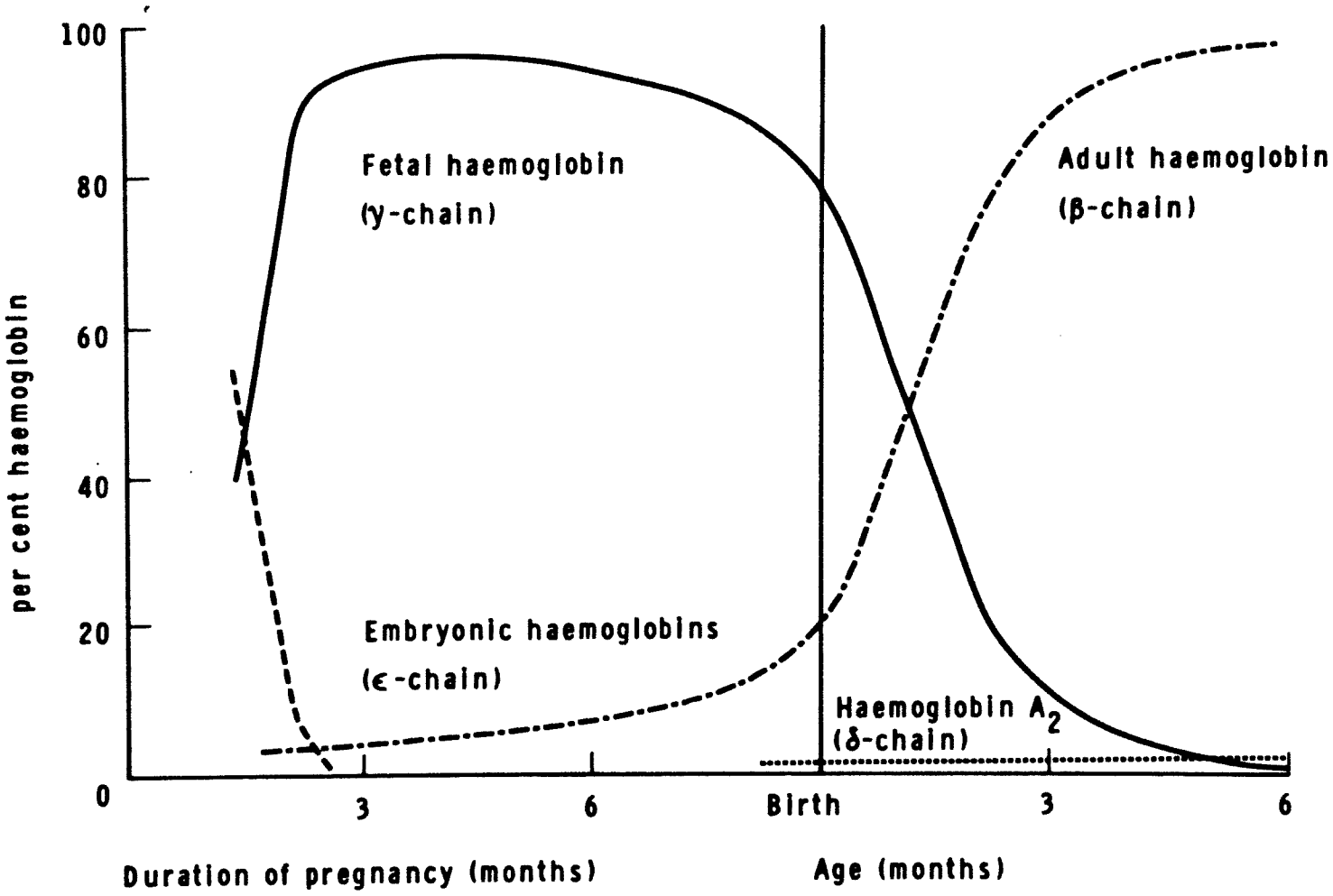


Figure 6 Normal haemoglobin production in utero and in the period after birth (from Huehns and Shooter, 1965).

heterozygotes maintain a slightly raised level of Hb F throughout adult life (Weatherall and Clegg, 1972).

These findings suggest that Hb F might play a possible protective role in falciparum malaria. This has a number of attractions in terms of the problems that have already been outlined concerning the mechanisms of resistance to malaria at the erythrocyte level. Firstly, since the host red cell in most cases of malaria contains Hb A, Hb F could present to the parasite a haemoglobin which could be regarded as abnormal as in the case of Hbs S, C or E. Secondly, if the slow fall off in levels of Hb F from birth were found to be present in all or the majority of these heterozygotes, it would provide a unifying mechanism of protection for these widely diverse diseases. This protective factor would moreover be present in those heterozygotes at six months to about one year, at a time when transplacental passively transferred immunity is at its lowest and actively acquired immunity is not fully developed. This coincides with an increase in the incidence of malaria (Garnham, 1949) and the age when mortality from the disease is at its highest (Bruce Chwatt, 1952; Colbourne and Edington , 1954). Finally, since Hb F is present for only a short time in the life of both the normal individual and in heterozygotes for the haemoglobinopathies, it would minimize the selective pressure for the emergence of a mutant parasite variant capable of flourishing in cells containing Hb F. Most of the population responsible for the maintenance of transmission of the disease from mosquito to mosquito would still have cells containing Hb A.

The structure and function of Hb F

Haemoglobin F is the main respiratory pigment of intrauterine life (Lorkin, 1973; Weatherall et al, 1974). It differs structurally from Hb A in one of the two pairs of peptide chains which constitute the globin moiety. Thus instead of the two α and two β chains of Hb A, Hb F has two α and two γ chains. Each γ chain differs from the β chain in 39 of the 146 amino acids that constitute each chain. In particular the γ chain has four isoleucine residues whereas the α and β chains (and thus Hb A) have none.

These structural changes in Hb F give rise to some important functional differences from Hb A. The most outstanding of these is the relatively high affinity Hb F has for oxygen when compared to Hb A. This is thought to be largely due to the substitution of histidine at position 143 in the β chain by serine in the γ chain. This replacement of histidine removes two positive charges and since negatively charged 2,3 diphosphoglycerate (2,3 DPG) is thought to bind partly at this site, Hb F has a lower affinity for 2,3 DPG. Since 2,3 DPG stabilises the deoxy form, Hb F with its low affinity for 2,3 DPG has a greater tendency to stay in the oxy form, that is, it has a higher affinity for oxygen.

Another property of Hb F is its relative resistance to denaturation by acids or alkalis. Application of this property has been made use of in the acid elution technique of Kleihauer (1957) which distinguishes cells containing varying amounts of Hb F from each other and from cells containing other haemoglobins. In this method, blood smears

are first fixed in ethanol and then placed in an acid buffer which elutes most known haemoglobins except Hb F which can then be stained. A whole range of cells, from those fully eluted to those staining darkly can then be distinguished if the Hb F is so distributed.

The possible role of Hb F in protecting human red cells from falciparum malaria prompted a preliminary investigation into the distribution of parasites amongst cells containing Hb F or Hb A. Children aged 3-6 months who are in the process of switching from fetal to adult haemoglobin possess red cells with largely one or the other haemoglobin or a mixture of both haemoglobins. Blood smears were obtained from such children infected with P. falciparum from Dr. D. Smith, Kisumu, Kenya and stained by the acid elution method of Kleihauer (1957). Examination of these smears revealed a paucity of parasites in the Hb F$^-$ containing cells (Plate I). There were clearly many possibilities to explain this observation and this opened many exciting avenues of investigation which were felt to be worth pursuing.

AIMS OF THE PRESENT STUDY

To examine the problem fully it soon became evident that an in vitro technique was required to observe the invasion and development of P. falciparum parasites in various types of red cells in more detail. The available technique (Phillips et al, 1975) for the culture of parasites through two generations in vitro at relatively high parasitaemias was not entirely suitable. Modification of such a technique was thus an important part of this study.

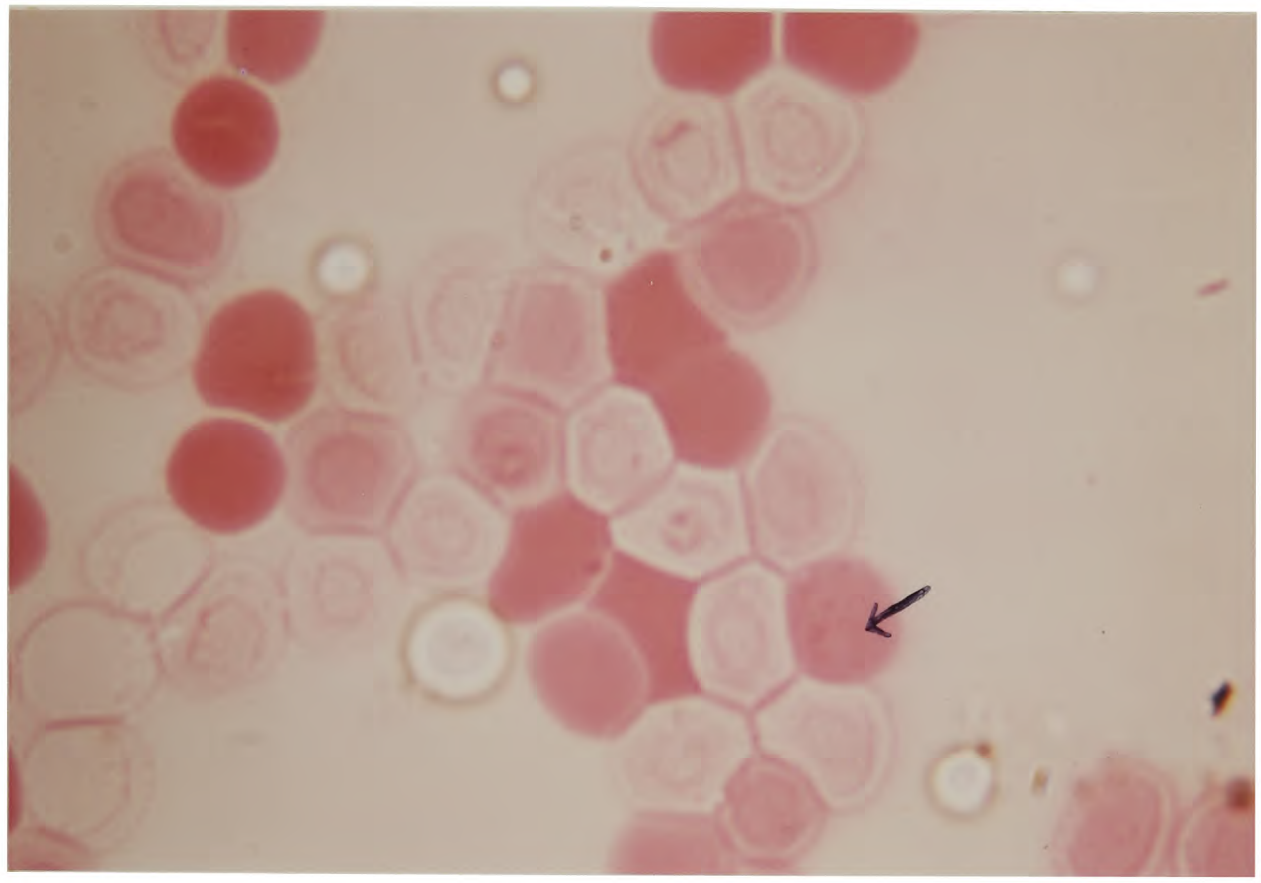


PLATE I Acid elution preparation of blood infected with P. falciparum and stained with haematoxylin and eosin as used in the Kleihauer method. The arrow indicates a parasite in a darkly stained Hb F-containing cell which can be easily missed (x 1500).

Before undertaking more detailed experiments it was first necessary to confirm the finding of relatively few parasites in the Hb F containing cells of young infants since only a few smears had been studied. In addition, the possibility existed that the staining of cells containing Hb F might obscure the presence of a parasite inside such a cell. Efforts could then be directed to the central hypothesis, namely that Hb F may modify the susceptibility of human red cells to P. falciparum. If evidence were to be found to support this hypothesis, it would clearly be important to attempt to determine whether this interference was mediated during the invasion of a parasite into a red cell, during development of the parasite within the red cell or whether the parasitised cell was predisposed to premature destruction. Moreover, with a suitable in vitro technique to examine parasite invasion and development, similar investigations could be carried out in cells from patients heterozygous for various haemoglobinopathies.

The work described divided itself conveniently so that each field trip attempted to answer a specific problem. Thus, the first visit examined the distribution of parasites between the Hb F-and Hb A-containing cells in infants, the second dealt with the invasion of parasites into cells containing Hb F, the third centered around the growth of the parasite inside such cells, whilst the last visit examined the relationship of the parasite and the red cell in other red cell disorders.

MALARIA RESEARCH IN SUBTROPICAL AFRICA

Most of the work presented in this thesis was carried out at the laboratories of the Medical Research Council (M.R.C.) in the Gambia, West Africa, during the rainy seasons of 1974, 1975, 1976 and 1977. This country was formerly a British colony of the same name and gained its independence in 1965. The Gambia, its name prefixed by the definitive article as it is derived from the name of the river which flows centrally through it, is situated on the bulge of the West African coast, surrounded on three sides by Senegal. It lies at a latitude of 13^{O} north of the Equator and the climate is subtropical with an annual average rainfall of about 113cms at the coast where these studies were conducted (Hutchinson, 1977). The rainy season extends from July to early November and it is at this time that the incidence of malaria is at its highest. This area is nevertheless endemic for malaria (McGregor et al, 1965) in that the spleen rate (the proportion of children aged 2-10 years with enlarged spleens) is near 75%, whereas the adults in this community are highly immune to the disease and the spleen rate is low. P. falciparum malaria is by far the most common form of human malaria encountered, whereas P. ovale and P.malariae infections are only occasionally seen. P. vivax infections are extremely rare in West Africa (Bray, 1958). During the rainy season,almost all young children under five years of age are found at some occasion to have falciparum parasites in their blood.

The M.R.C. has laboratories situated on approximately 90 acres of land on the coast at Fajara, about 10 miles from the capital Banjul (formerly Bathurst). Despite the difficulties encountered due to the relative isolation, environment and climate, this unit remains one of the finest in West Africa. Electricity for the laboratories comes from the main power station at Banjul, but if this should fail, stand-by generators at the unit cut in automatically. The laboratories are continuously air conditioned 24 hours a day, maintaining a temperature of about 23^{o}C and a humidity between 50 and 60%.

The outpatient clinic deals with over 1000 patients each month. These patients come predominantly from the nearby villages but some come from far afield, even from neighbouring Senegal to seek medical help. Very few patients take malaria prophylaxis and it is only very seldom that patients have taken antimalarials when first seen. This is surprising since no prescription is necessary to get any drug over the counter at the pharmacies. The unit also has a 36-bed hospital ward for the treatment of particularly sick patients.

It is very difficult to divorce the work described in the following pages from the setting in which it took place. But more than the place itself, it is the people of the Gambia whose friendliness, warmth and helpfulness remains unforgettable, and who provided the environment in which such work could take place so pleasantly and effectively.

CHAPTER 2

METHODS AND THEIR EXPERIMENTAL ASSESSMENT

(All materials used and their preparation are described in Appendix A).

A. METHODS OF PARASITE CULTURE

Introduction

A major restriction in the examination of malarial parasites and red cells from various disorders has been the failure to develop a suitable system for the *in vitro* culture of such parasites, in particular for *P. falciparum*. Raper (1959) and Luzzatto *et al* (1970) have done some preliminary *in vitro* work with this parasite inside various red cell types but their cultures contained relatively few parasites and were short-lived. Essentially what was required was a system which could maintain a reasonably heavy infection of parasites for at least two generations *in vitro*.

The first reports on successful culture of human malarial parasites were by Bass and Johns (1912). They cultivated *P. falciparum* in a medium composed of dextrose and human serum in stationary glass test tubes. In order to maintain more than one generation of parasites they first removed the buffy coat from the infected blood "to avoid destruction of the parasites by the white cells at the time of segmentation." However, their work suffers from the disadvantage that it is purely descriptive and no quantitative data are presented. Nevertheless, these

studies are remarkable since it was not until 60 years later that Diggs et al (1971) were able to equal in any measure their success with regard to consistent increase in parasite numbers in vitro.

In the intervening period many combinations of different methods were attempted but these were not entirely successful. Although improvements have been made there has been no single factor which has resulted in successful parasite culture. Recent advances have paid attention to multiplicity of factors such as the composition of the medium, density of parasite infection, the origin and age of red cells, plasma or serum factors and buffers, white cell removal and temperature requirements, and the vessels for culture.

The use of a proprietary tissue culture medium for the culture of malaria parasites was reported by Trigg (1969b). He showed equal, if not better growth of P. knowlesi with Medium 199 than with expensive and tediously prepared laboratory Harvard medium (Ball et al, 1945; Geiman et al, 1966) which had been the main culture medium used. Even the ingredients in Medium 199 were far in excess of parasite requirements and Trigg and Gutteridge (1971) described a minimal medium in which P. knowlesi were successfully grown. This medium contained a balanced salt solution, glucose, isoleucine, methionine, adenosine, para amino benzoic acid (PABA), biotin and calcium pantothenate. Together with plasma this medium gave better results than the Harvard medium. However it was only in 1976 that Medium 199 was shown to be superior to Harvard medium for culture of P. falciparum

(Mitchell *et al*, 1976).

In all attempts to culture malarial parasites, plasma or serum has been found to be an indispensible component of the medium. Although Siddiqui *et al* (1967) replaced plasma with a single fatty acid, stearic acid, Trigg (1969a) found that the increase in parasite numbers and glucose utilised under these conditions did not reach the levels obtained in the presence of whole plasma. The importance of plasma or serum has always been thought to be the provision of some still unidentified growth factor, but just as important has been its function as a buffer for the system (Trigg, 1969a). Over and above this, all media that have been used with success have incorporated in addition a bicarbonate/carbon dioxide buffer system which maintains the medium at a pH of 7.4 and allows for CO_2 fixation by the parasite (Fletcher and Maegraith, 1972).

Other improvements in parasite culture techniques have paid attention to both parasites and the red cells used. Thus the highest rates of invasion *in vitro* have been found by initiating cultures with the most mature parasites found in the peripheral blood, which in *P. falciparum* infections are the late ring forms (Trigg, 1969b; Trager, 1971). Furthermore, assessment of merozoite invasion and subsequent growth becomes easier and more accurate by using samples where all the parasites present are in the same stage of development (synchronous). The condition of the red cells used in culture is also important since they are known to develop metabolic abnormalities within hours when incubated *in vitro* at $37^{o}C$ (Gomperts, 1967). A recent paper by Trigg and Shakespeare (1976) demonstrated that rhesus monkey

erythrocytes preincubated *in vitro* at 37°C are less susceptible to invasion by merozoites of *P. knowlesi* than are erythrocytes freshly obtained from the monkey. However, incubation of normal monkey erythrocytes for up to 48 h *in vitro* did not affect the ability of these uninfected red cells to metabolise glucose either via glycolysis or via the hexose monophosphate shunt. There was also no change in the ATP levels of these incubated erythrocytes.

In an attempt to improve methods many workers have designed elaborate systems for the perfusion of the medium (Geiman *et al*, 1946). However, these are cumbersome and difficult to replicate, especially when involved in work in remote parts of the world. Simple dilution techniques where the parasites are diluted in relatively large amounts of medium are more convenient and can be improved by retrieval of the cells and subculture with fresh medium and red cells (Phillips *et al*, 1972; Mitchell *et al*, 1976). Using this method Phillips and his co-workers were able to subculture the parasites of *P. falciparum* through almost three cycles *in vitro* with consistent marked increase in numbers during the first subculture, but only maintenance of numbers in the second subculture. Phillips *et al* (1975) reported a further improvement by adapting their early methods to culture in microtissue plates. Thus they were able to screen large numbers of human sera for anti-*P. falciparum* activity. These methods of Phillips *et al* (1972, 1975) were the ones available when this study was first undertaken. At the time they were probably the most successful reported. They were particularly suitable because the microtissue culture plate technique allowed a large number of different blood

samples to be tested at the same time.

Whilst the present study was being carried out, Trager and Jensen (1976) and Haynes et al (1976) independently reported the successful continuous culture of P. falciparum in vitro. They both used proprietary culture medium and human sera, but high levels of CO_2, low levels of oxygen and maintenance of the numbers of infected cells at 1% or less were special features of their methods. However, parasites lost their synchronicity in culture. This factor, in addition to the low percentage of infected cells involved, makes their method seem less suitable for observation of red cell invasion and subsequent growth in vitro. On the other hand, one possible use of their continuous culture techniques would be the examination of long term parasite survival in red cells from various disorders.

The methods used in this study for culture of P. falciparum follow those of Phillips et al (1972, 1975) but in addition incorporate a means of removing white cells and concentrating schizont-infected red cells. The method also differs in the addition of a further buffer to maintain physiological pH and in the proportion of cells, medium and AB serum mixed together. Some of these modifications will be described below.

Addition of a further buffer to the medium

The addition of plasma or serum and the CO_2/bicarbonate buffer system have traditionally been used for parasite culture. However more recently it has been claimed

that the organic sulphonic acid buffers HEPES[1], BES[2] and TES[3] are superior to the bicarbonate buffer system in the cultivation of mammalian cells (Eagle, 1971). Siddiqui and Schnell (1973) therefore maintained cultures of P. falciparum buffered with only HEPES, BES or TES each at a concentration of 40 mM. They found these to be superior to both glycylglycine or no buffer at all but unfortunately did not compare them with the CO_2/bicarbonate system.

It was felt in this study that the organic sulphonic acid buffers should be of particular benefit at the commencement of the cultures and during the period of removal of the cells from the gased environment when the bicarbonate buffer system was not effective. However, since the bicarbonate requirement of parasites is unknown and they are thought to fix CO_2 (Fletcher and Maegraith, 1972), both the bicarbonate buffer system and 10 mM HEPES were used to buffer the culture medium.

Stationary versus moving cultures

Several methods previously used have agitated malarious blood in culture by means of rocking or rotation (Geiman et al, 1946; Williams and Richards, 1973) and others have used continuous flow techniques to change the medium over settled red cells with or without an intervening membrane (Trager, 1971). The majority of workers, however, have used simple stationary dilution techniques and have replaced the medium when necessary

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1 HEPES N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
2 BES N,N'-bis(2 hydroxyethyl)-2-aminoethanesulphonic acid
3 TES N-Tris (hydroxyethyl) methyl-2-aminoethanesulphonic acid

(Phillips et al, 1972; Trigg, 1976). Apart from the simplicity of stationary cultures it seems that the rates of invasion of merozoites are considerably higher under these conditions but no data are available. Bass and Johns (1912) noted that any disturbance of their stationary cultures at schizogony caused "rapid deterioration of the parasites and poor reinvasion". McKee (1951) commented on the high frequency of multiple infected cells in stationary cultures. Therefore a compromise was made in most of the experiments in this study:- where the large culture flasks were used, cultures were generally shaken but subculture was made into stationary microtissue culture trays. This was to optimise the beneficial effect of mixing the medium during growth and allowing maximal invasion to occur when the schizonts ruptured and the merozoites released were invading new cells.

Overall scheme of culture methods

Parasitised blood was freed of white cells and grown to mature schizont stage in large 2 L Erlenmeyer flasks (Fig.7). At the end of this period these infected (donor) cells were concentrated, mixed with the uninfected (recipient) cells to be tested and subcultured in microtissue culture trays. In certain experiments where only the conditions of culture were being tested, infected blood could be plated directly into microtissue trays after removing the homologous plasma by washing the blood twice with phosphate buffered saline (PBS - Appendix A) and resuspending in AB serum. Infected cells tend to agglutinate if grown in homologous plasma (Siddiqui and Schnell, 1970).

Removal of white blood cells

Bass and Jones (1912) stressed the need to remove

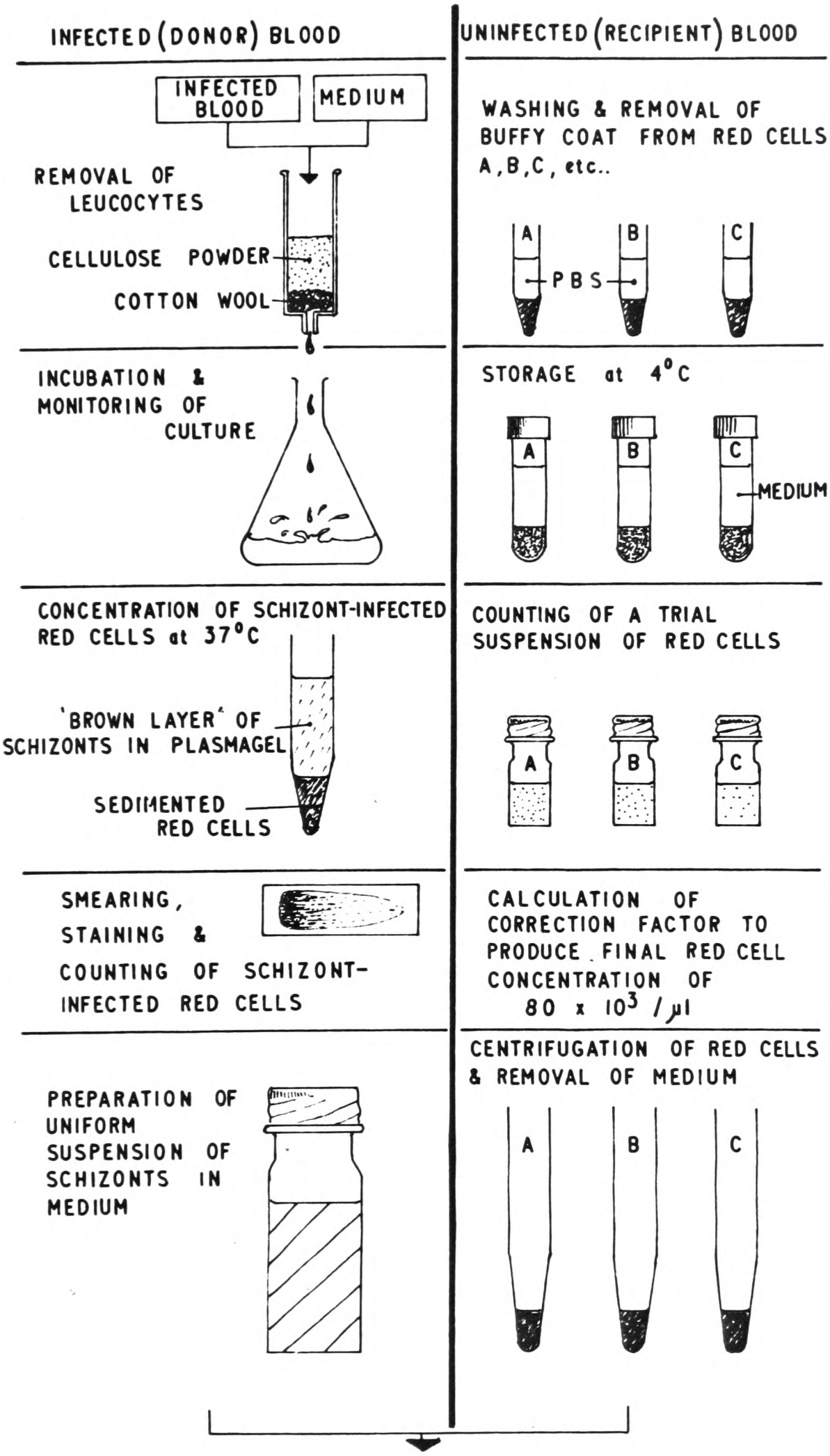


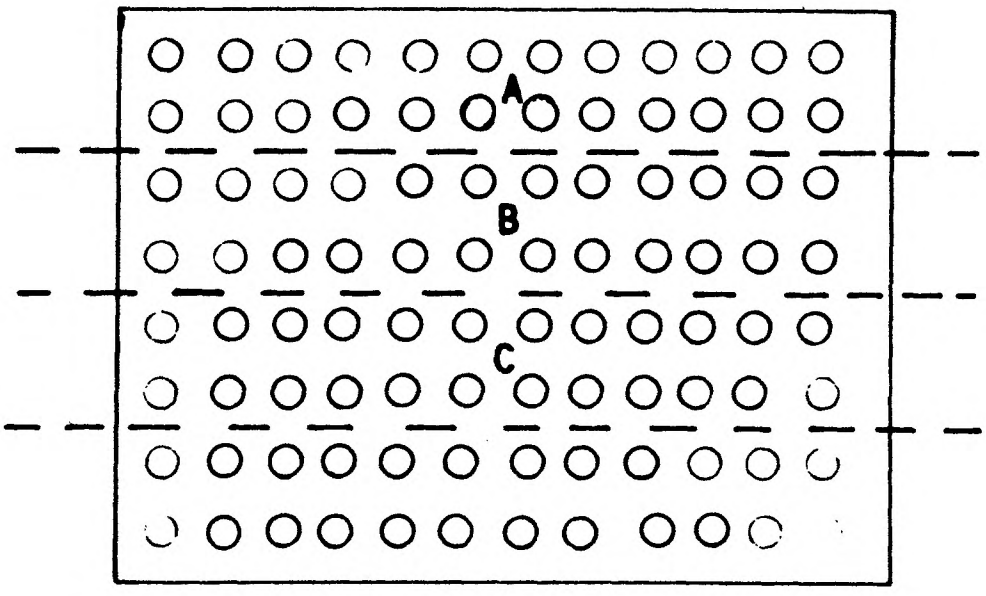
Figure 7 Diagram showing the subculture method of growing P. falciparum

MIXING OF DONOR SUSPENSION & RECIPIENT CELLS



EACH MIXTURE : INFECTED CELLS 2 - 5 %
RECIPIENT CELLS 80 x $10^3/\mu l$

PLATING OF MIXTURE INTO MICROTISSUE CULTURE TRAY



INCUBATION at 37° C

ASSESSMENT OF INVASION RATE , DISTRIBUTION & DEVELOPMENT OF PARASITES

Figure 7 (cont) Subculture method

white cells from long-term cultures, believing that these cells interfered with the invasion of red cells by merozoites. White cells can also complicate the assessment of parasite growth as measured by the incorporation of radioisotopes (Williams and Richards, 1973; Richards and Williams, 1973; Trigg, 1976). Removal of leucocytes from infected blood in this study was found to be particularly important as the method used to concentrate parasitised cells also concentrated white cells and therefore contaminated such schizont infected red cell preparations.

Various methods are available for the removal of white cells from whole blood infected with malarial parasites, for example the removal of the buffy coat (Beutler, 1971), sedimentation in dextrose (Langer et al, 1967; Levy and Chou, 1973) and in glucose/sucrose gradients (Williamson and Cover, 1966). However one of the simplest methods has been the use of cellulose powder, first used to remove leucocytes from uninfected whole blood (Fulton and Grant, 1956). Richards and Williams (1973) showed that by using this powder, parasites of P. Berghei were still viable and could be further cultured after removal of white cells. Mitchell et al (1976) used CF 11 cellulose powder in P. falciparum infections but for another purpose, namely to retrieve red blood cells containing mature forms of the parasite from culture. Red cells containing these mature forms, unlike those with the ring forms, are entrapped by cellulose. Homewood and Neame (1976) compared CF 11 cellulose powder to other methods of white cell removal in P. berghei infections (i.e. buffy coat removal and dextrose, glucose and sucrose gradient

sedimentation) and found cellulose powder to be the most efficient. For this reason its use was attempted in these studies although it had not previously been used to gain viable ring forms of P. falciparum.

CF 11 cellulose powder (Whatmans) was first suspended in bulk in PBS and autoclaved. This was then shaken and poured into the barrel of a sterile 30 ml glass syringe, (the outlet of which was plugged with sterile non-absorbent cotton wool), to form a column of powder measuring approximately 6x2 cm. Five ml of infected blood containing ring forms of the parasite was poured onto the column and absorbed into the fibres. The blood was then filtered into a 2 L Erlenmeyer flask by washing the column with supplemented Medium 199 (Appendix A). After washing, the column could be seen to be nearly free of red cells. The flask containing the blood in 100 ml medium was then gassed with a 5% CO_2 in air mixture, stoppered, and incubated at 37°C with gentle shaking (Fig. 7). Smears of the blood were routinely made before and after passing through the cellulose powder. The red cells were largely free of white cells as judged by the smears. Electronic cell counting was unfortunately not possible with these samples as contamination with small pieces of fibre invalidated the results. There was also no decrease in the percentage of small or large ring forms (Table 1a). In some cases it was noticed that parasites in the red cells emerging from the column appeared to be localised peripherally in the red cells and that these parasites sometimes had an irregular outline. This may have been artefactual since even in these cases the parasites

	Total Red Cells Counted	No. of Schizonts	No. of Rings	Schizonts %	Rings %	Enrichment ($\frac{\%\text{ Schizonts post concentration}}{\text{"}\quad\text{"}\quad\text{pre}\quad\text{"}}$)
Pre Concentration:-						
Pre CF 11	580	0	29	0	5.0	-
Post CF 11 (0 h)	759	0	40	0	5.3	-
Post CF 11 (24 h)	1213	65	21	5.4	1.7	-

Table 1a The effect of removal of white cells by cellulose powder on parasite numbers and subsequent parasite development.

reached the schizont stage in culture with no abnormal forms.

Unfortunately, no information is available on the effect of the cellulose powder on red cells and in particular on the red cell membrane. Thus in all cases white cells were removed from the uninfected (recipient) cells by centrifugation followed by pipetting of the buffy layer.

Monitoring of the cultures

The parasites were usually grown *in vitro* to the mature schizont stage, taking on average about 24 h. Growth was assessed during this period by sampling and preparing a blood film after about 12 h in culture. An example of such a culture is shown in Plate II. Following this sampling the cultures were each supplemented with 2 ml of 10% glucose and 1 ml of 6.6% sodium bicarbonate. After a further period of growth when the majority of parasites had grown to mature schizonts, the cultures were again disturbed in order to concentrate the infected cells. In the case documented (Table 1 and 2) both schizont and small ring forms were present, though in most cases cultured parasitised cells were concentrated before any schizonts had ruptured and given rise to ring forms. In this particular case the presence of both young and mature forms was desirable in order to examine the effects, if any, the subsequent method of concentration of infected cells would have on the various forms of the parasite.

Concentration of schizont infected cells

One major contribution to the culture methods was a means whereby the schizont-infected cells of *P. falciparum*

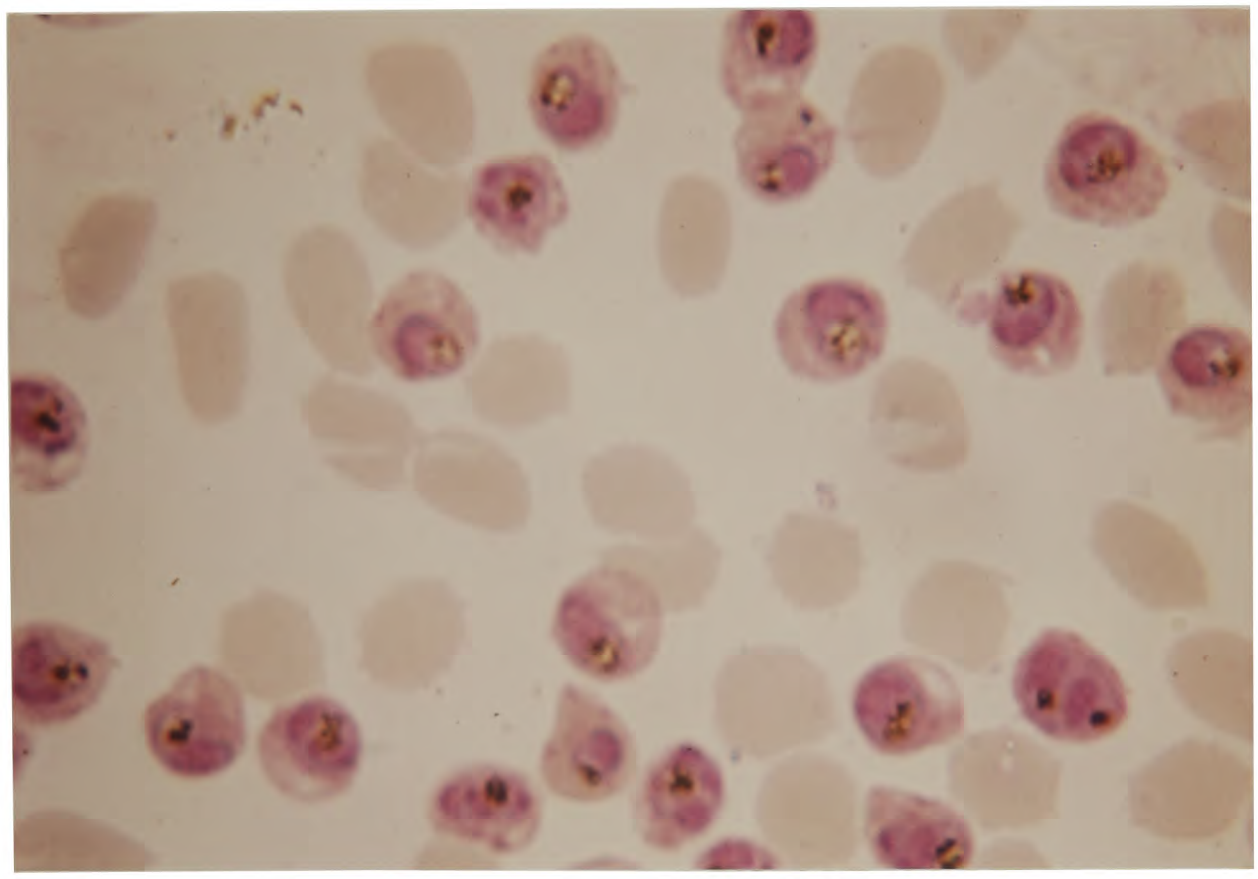


PLATE II Giemsa stain of a typical culture of heavily infected blood with the majority of parasites in the late trophozoite stage (x 1700)

were concentrated, so that they were still suitable for further *in vitro* culture. This was essential so as to minimise the number of uninfected donor cells present after mixing with the recipient cells.

Numerous methods for the concentration of cells infected with *P. falciparum* have been devised in the past. These include centrifugation (Bass and Johns, 1912), retrieval from infected placentae (McGregor *et al*, 1966) and more recently differential sucrose gradient centrifugation (Williamson and Cover, 1975), a method which had previously been used on P. knowlesi (Williamson and Cover, 1966). Zuckerman *et al* (1967) used dextran sedimentation but only on the early ring forms of *P. falciparum*. These methods have been largely used to produce purified parasite material with antigenic activity and the viability of the parasites for further *in vitro* culture has not been assessed fully. Since the degree of concentration of parasites by centrifugation is only three- to four-fold, alternative methods were sought.

Plasmagel, a solution consisting of 3% w/v gelatin has been previously used to concentrate lymphocytes from human blood (Coulson and Chambers, 1964) and it was thought possible that this might concentrate red cells infected with *P. falciparum* once the white cells had been removed.

Therefore when the parasites had grown in the culture to the schizont stage, the red cells were separated from the culture medium by centrifugation at 600 g for five minutes and reconstituted to their original volume (5 ml) with Medium 199 in a 15 ml centrifuge tube. Half this

volume (2.5 ml) of Plasmagel (3% w/v gelatin - Laboratoire Roger Bellon, Neuilly) at 37°C was mixed with this red cell suspension which was then allowed to stand at 37°C for 15 minutes (Fig. 7). Two distinct phases could be seen at the end of this period with concentration of schizont infected cells in the upper layer. In the experiment tabulated, the optimum volume of Plasmagel and time of sedimentation were ascertained (Table 1b and 2). The highest concentration of schizonts was obtained at a red cell to Plasmagel ratio of 2:1 (Table 1b). At this ratio the concentration of parasites increased with time of sedimentation up to 15 minutes; thereafter the gain was minimal (Table 2). If left long enough, 100% parasitised cells could be obtained but this may be at the expense of the viability and number of parasites recovered. Photomicrographs of typical cultures before and after concentration with Plasmagel are shown on Plates III and IV respectively.

The results in both tables indicate that the ring forms from the next generation of parasites are not concentrated by this method. This is presumably because they do not markedly alter cell buoyancy.

The use of Plasmagel provides a simple, rapid and consistent method which required minimal physical manipulation to concentrate schizont-infected cells. In most of the experiments, the percentage of infected cells concentrated was between 70 and 90%. In these respects, and also because the parasites obtained were consistently capable of invading and developing inside fresh red cells added to the culture,

	Total Red Cells Counted	No. of Schizonts	No. of Rings	Schizonts %	Rings %	Enrichment ($\frac{\%\ \text{Schizonts post concentration}}{\text{"}\quad\text{"}\quad\text{pre}\quad\text{"}}$)
Post Concentration:-						
Red cell suspension to Plasmagel ratio:						
1 : 0	613	24	16	3.9	2.6	0.7
2 : 1	508	418	0	82.3	0	15.2
1 : 1	629	369	3	58.7	0.5	10.9
1 : 2	547	123	11	22.5	2.0	4.2

Table 1b The effect of red cell suspension to Plasmagel ratio on the concentration of red cells infected with schizonts of P. falciparum.

Sedimentation Time (minutes)	Total Red Cells Counted	No. of Schizonts	No. of Rings	Schizonts %	Rings %	Enrichment
Pre-concentration	637	27	32	4.2	5.0	-
5	581	212	16	36.5	2.8	8.7
10	533	348	5	65.3	0.9	15.5
15	557	427	7	76.7	1.3	18.3
20	540	418	2	77.4	0.4	18.4
120	100	100	0	100.0	0.0	23.8

Table 2 The effect of increasing sedimentation time on the concentration of schizont-infected cells in Plasmagel.

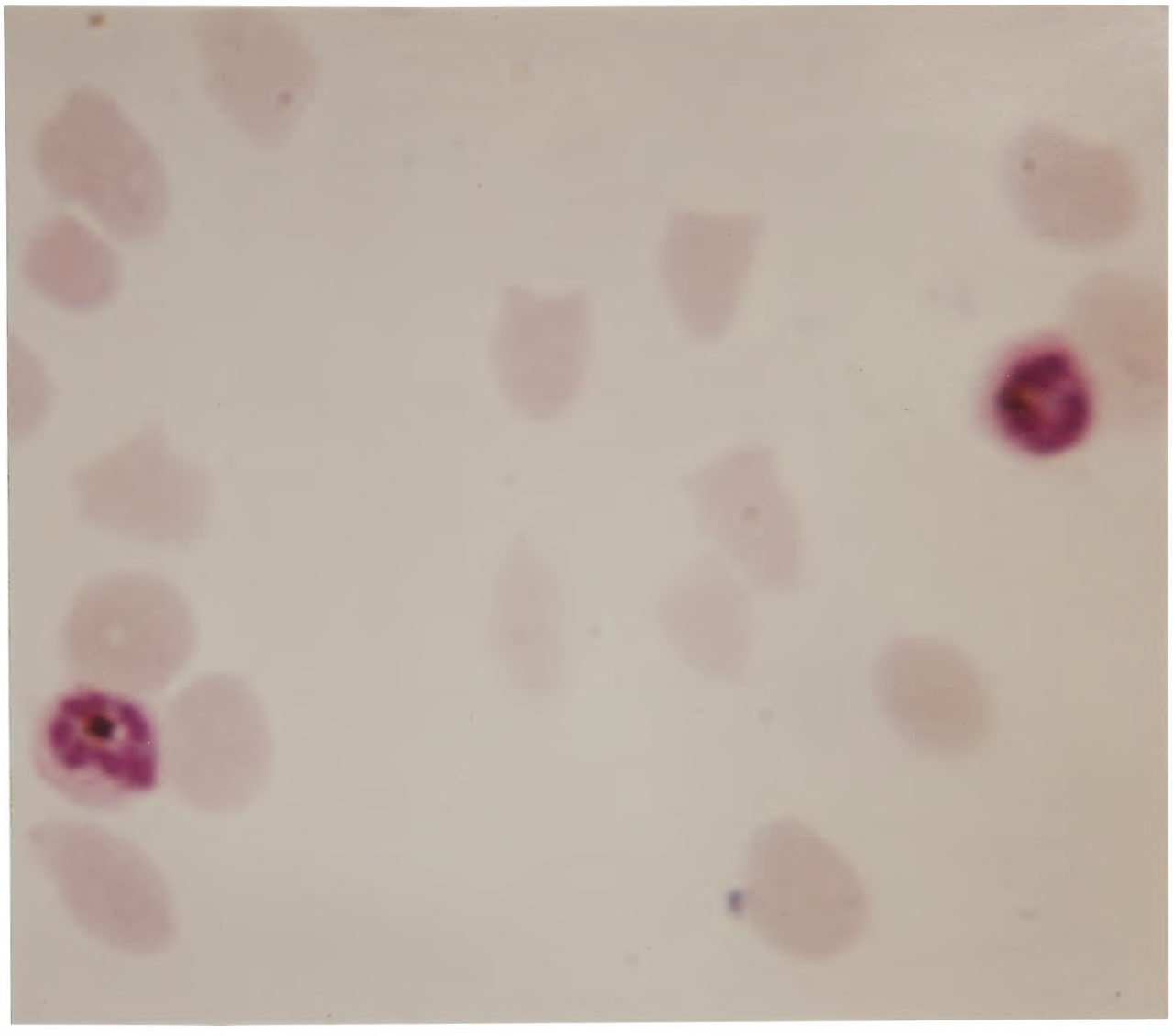


PLATE III Typical culture of P. falciparum showing two schizonts prior to concentration of infected cells (x 2500).

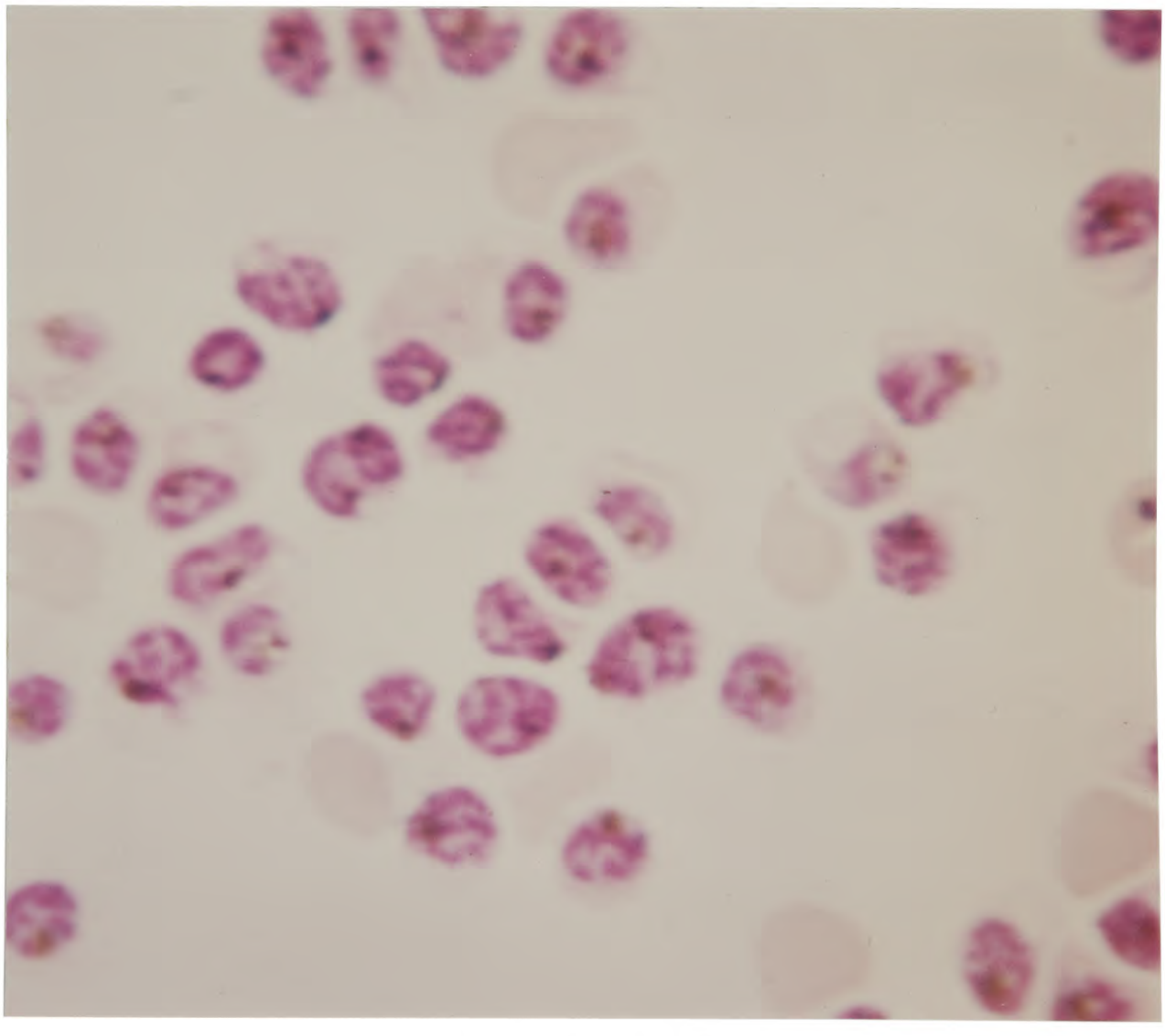


PLATE IV The same culture as in Plate III after concentration of schizont infected cells with Plasmagel (x 1700)

this new method has distinct advantages over the existing ones. Another important feature of this method is that it does not concentrate smaller ring forms of the parasite if they are present. This finding not only aids synchronicity in subsequent parasite cultures but also supports the belief that the younger forms of P. falciparum do not appreciably alter cell buoyancy, a fact about which very little is known or remains undocumented(see page 79).

Subculture into microtissue culture trays (Fig. 7)

The parasitised red cells recovered from the upper layer after sedimentation with Plasmagel were recovered by centrifugation, a stained smear of them was prepared and counted for the percentage of infected cells present and without washing were suspended in a mixture of three parts supplemented Medium 199 and one part non-immune AB serum. The total volume of this suspension was sufficient for the required number of wells in the tray calculating 250 µl per well. This donor cell suspension was mixed gently to disperse the schizonts evenly throughout the medium so as to ensure a uniform inoculum of parasites. The infected suspension was then subdivided into aliquots to which uninfected cell types could be added.

The recipient cells were prepared and stored as outlined in the section on blood specimens. Each cell sample to be tested was added to an appropriate volume of parasitised red cell suspension so that the final concentration was approximately 80×10^3 cells/µl with 2-5% schizont-infected cells. This necessitated the counting of a sample specimen of uninfected cells with an electronic cell counter (Coulter model ZF) and calculating the volume of red cell type to be

added to give a concentration of $80x10^3$ cells/µl. This was particularly important when dealing with extremes in cell size, e.g. the microcytes of β thalassaemia or the macrocytes of cord cells. Finally, 250 µl of the mixture of donor and recipient cells was pipetted into each well of a microtissue culture plate and incubated in a 5% CO_2 in air mixture at 37^oC without shaking.

B. ASSESSMENT OF INVASION AND DISTRIBUTION OF PARASITES

To assess the rates of invasion, smears were made from the cultures as soon as possible after the schizonts had ruptured and given rise to a new generation of rings. The reason for early sampling was to minimise the possibility of differential loss of parasitised cells and was generally done 12-18 h after subculture when most of the parasites which had invaded the recipient cells were in the small ring (SR) form. The percentage parasites is the number of ring forms per 100 red cells and excludes any persisting schizont infected cells. Generally at least 1000 red cells were counted. Where preparations contained Hb A and Hb F, the modified method of Kleihauer was used, and cells were classified as follows: those fully eluted Hb A-containing cells (Adult - A), those with mixtures of Hb A and Hb F (Intermediate - I) and those with predominantly Hb F (Fetal - F) (Plate V). In such cases where the distribution of parasites in the various cell types was assessed, at least 2000 cells were counted on each smear. The ratio (R) between the percentage parasites in the test and the control cells was calculated so that the comparison could be made with invasion rates in other experiments.

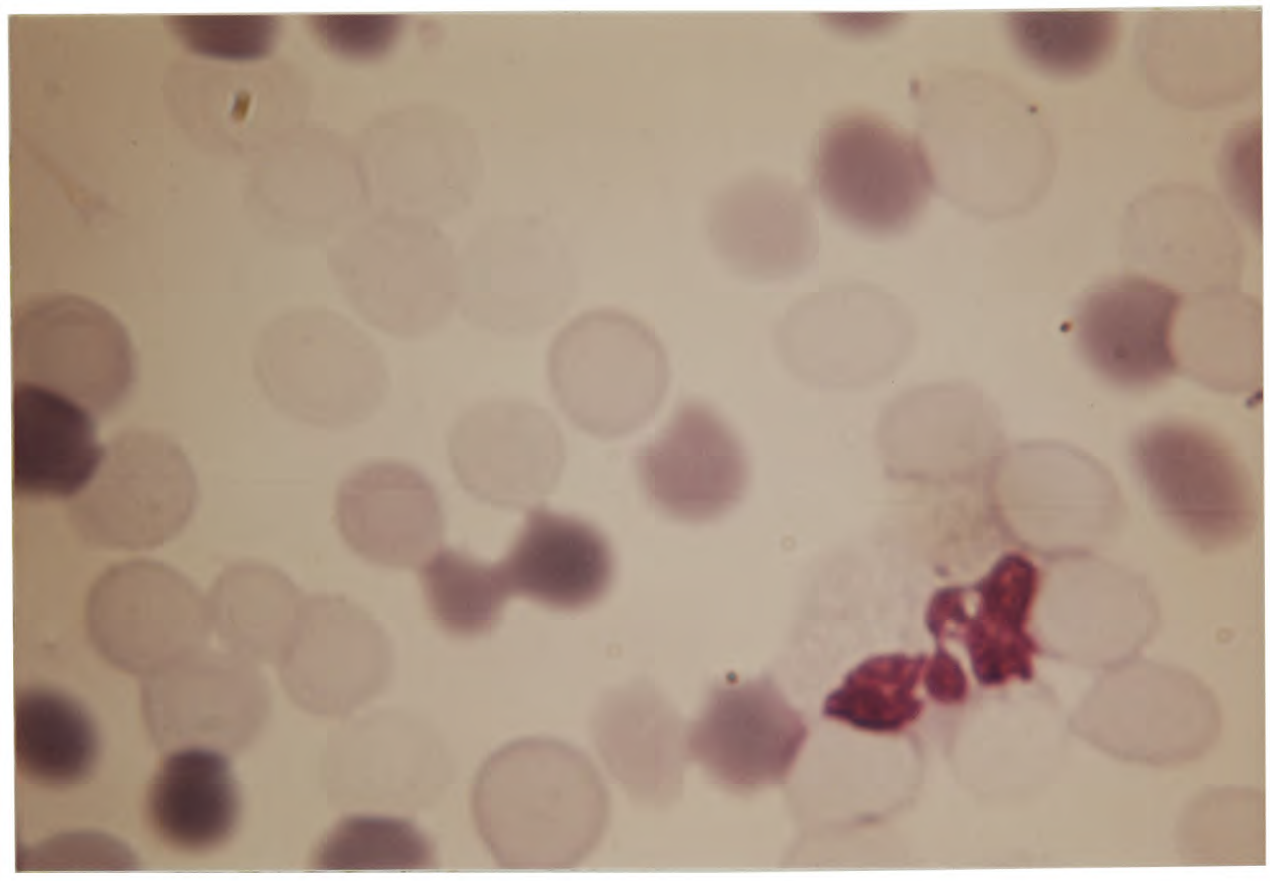


PLATE V Uninfected red cells stained by modification of the Kleihauer technique showing darkly staining F cells, intermediately staining I cells and the outlines of the fully eluted A cells (x 1700).

C. ASSESSMENT OF PARASITE DEVELOPMENT

Three methods to asses growth were used:

(a) Morphology.

(b) Isotope incorporation

(c) Weight of enlarged photographic cut-outs of individual parasites.

(a) Morphological assessment from stained smears

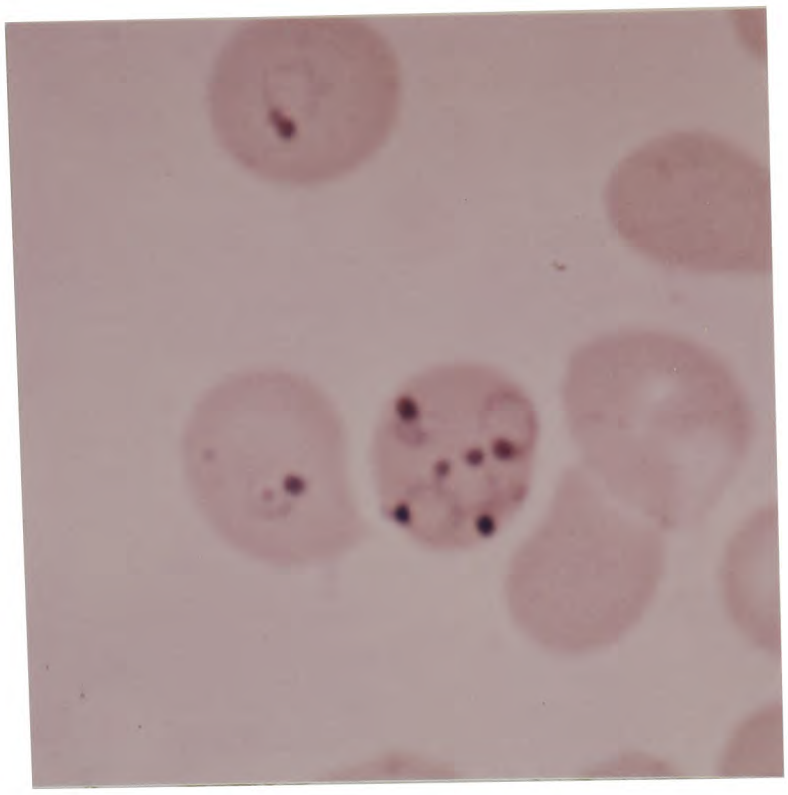
At the appropriate time after reinvasion (usually about 48 h after subculture) smears were made from the cultures to be compared. In each cell type, the parasites in 100 singly infected cells were classified into one of the stages shown in Table 3 which gives a description of each stage. These stages are also illustrated on Plate VI a - h.

As a descriptive aid to assess growth, an arbitrary scoring system was devised so that overall growth in each cell type could be expressed as a single figure which incorporated all the data on the stages of development of the asexual forms in a single culture. Thus, individual parasites were scored from 0 to 6 as shown in Table 3 and the total of these was divided by the number of asexual forms counted to give the mean growth score (MGS). Theoretically, this score could range from 0 to 6 and is given to one decimal place. The difference (D) between the MGS of the parasites in the test and control cells was calculated so that comparison could be made with growth differences of the asexual forms in other experiments. Where growth was better in the test cells than in the control, this difference (D) would be positive and vice versa. The maximum D between two cultures is six assuming that all the parasites

Stage of Development	Abbrev-iation	Description	Growt Score
Abnormal	A	Nucleus indistinguishable from cytoplasm. Pyknic hyperchromatic parasite with irregular outline	0
Small ring	SR	Parasite with a small nucleus and a thin rim of cytoplasm enclosing a relatively large vacuole. Approximate size < 2 μ	1
Large ring	LR	Increase in nuclear size and cytoplasm. Smaller vacuole. No pigment visible. Approximate size > 2 μ	2
Small trophozoite	ST	First appearance of pigment. Single clump.	3
Large trophozoite	LT	Large parasite (diameter of parasite exceeding radius of normocytic red cell). Larger nucleus and pigment clump.	4
Immature schizont	IS	Division of nuclear material (> 1 but < 4 nuclei)	5
Mature schizont	MS	Four or more discrete nuclei present.	6

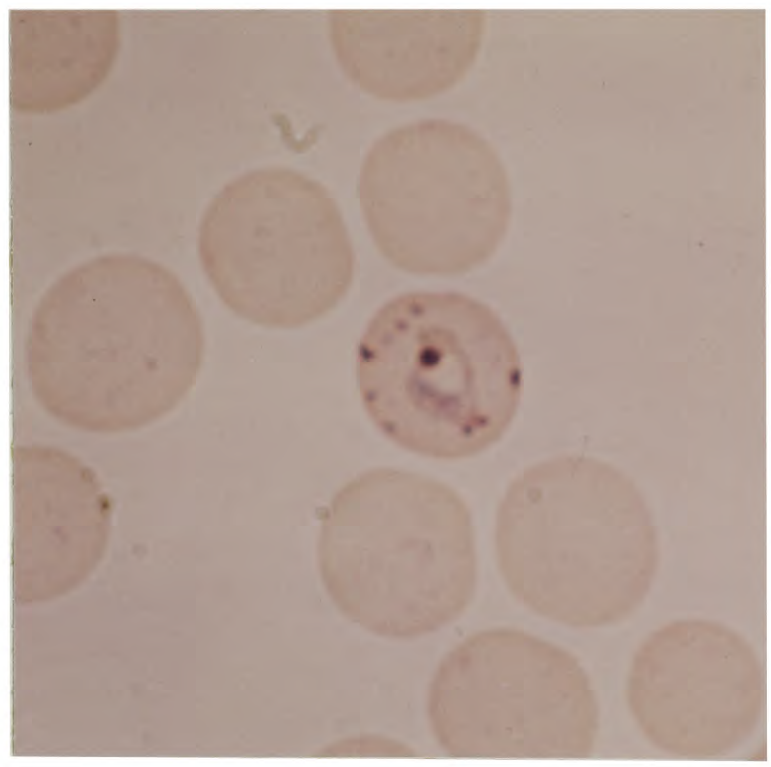
Table 3 Morphological assessment of the growth of P. falciparu

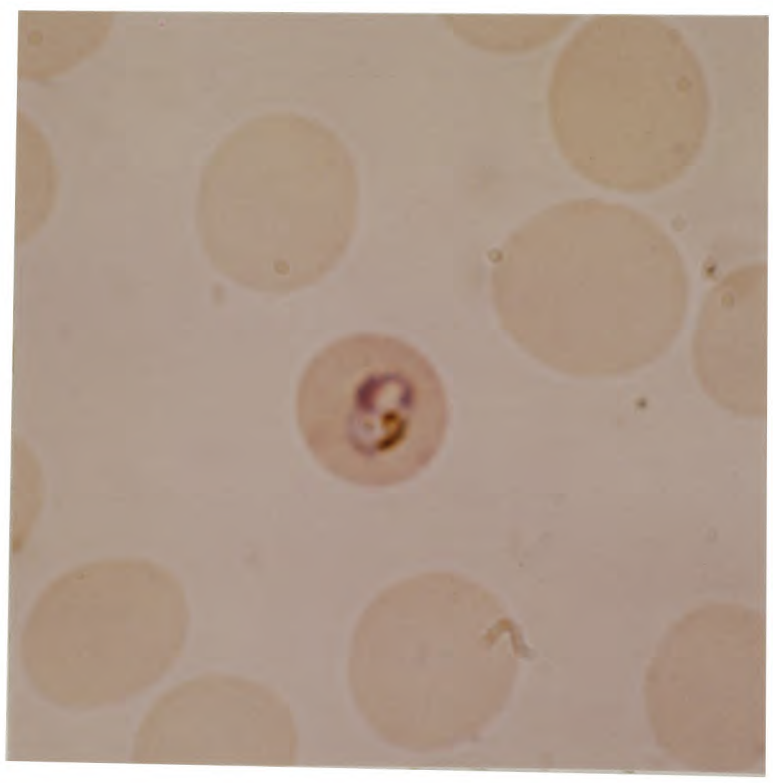
An arbitary scoring system was used to determine growth in any single culture and to compare it with other cultures in the same experiment. The parasites were scored 0 to 6 depending on their maturity and a mean growth score (MGS) was determined by calculating the sum of the individual scores of 100 parasites and then dividing by the number of parasites counted (i.e. 100).



a. Small ring SR

b. Large ring LR

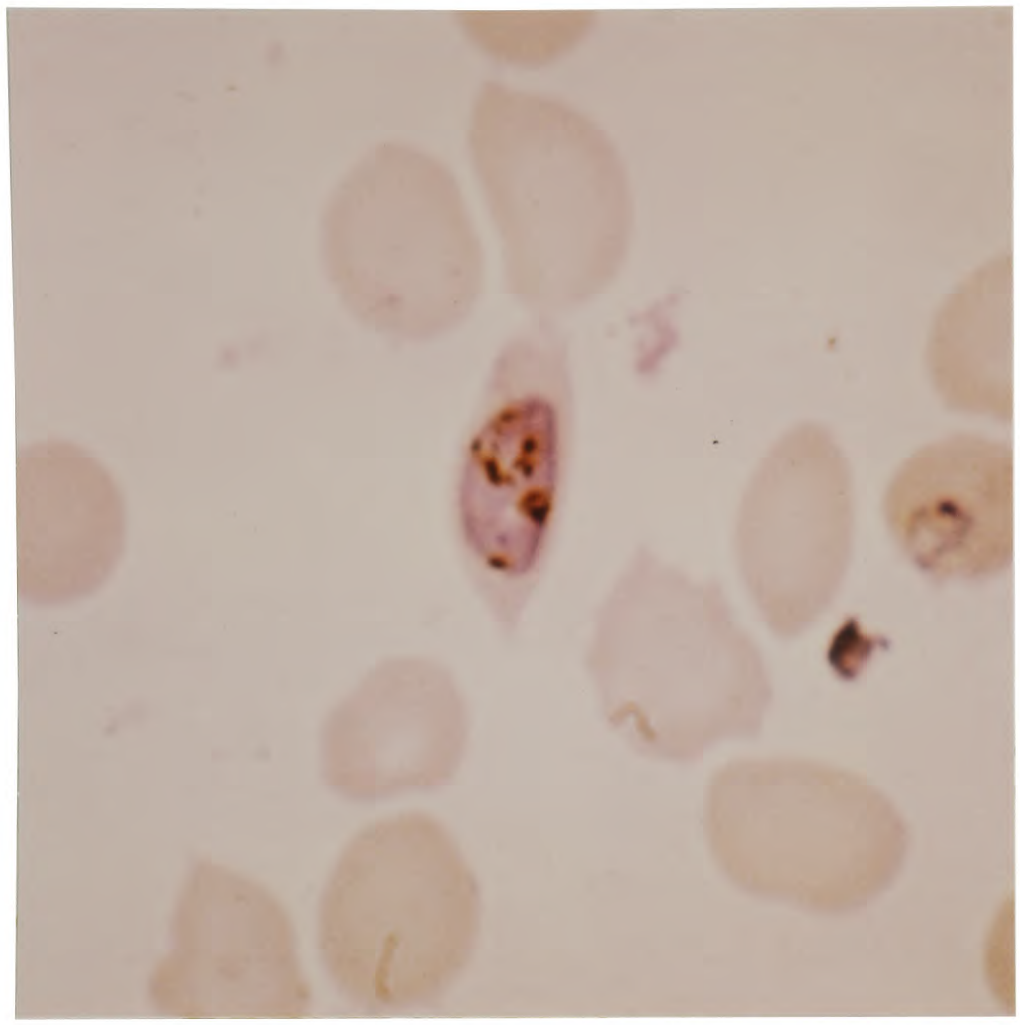




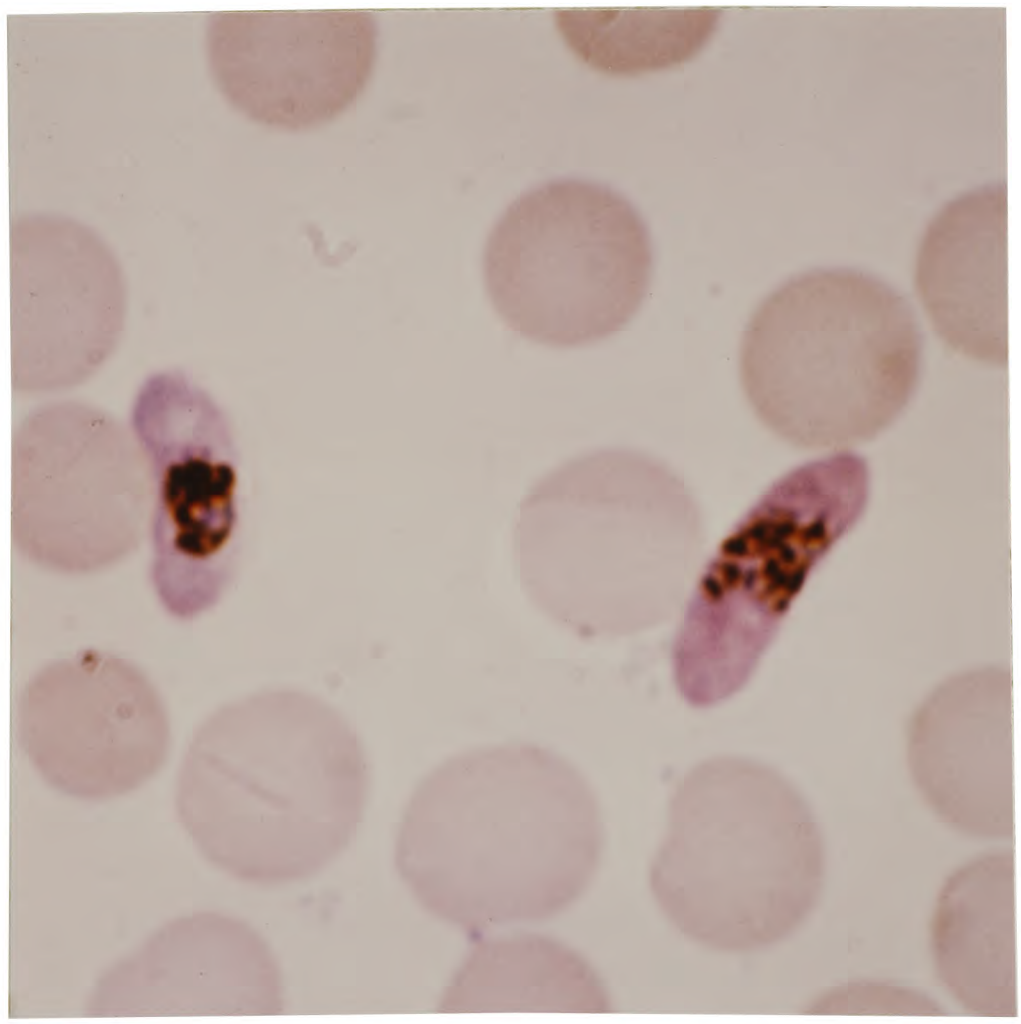
c. Small trophozoite ST

PLATE VI Morphological assessment of the development of P. falciparum (x 2500).

Asexual erythrocytic forms



g. Immature gametocyte



h. Mature gametocyte

PLATE VI (cont) Sexual erythrocytic forms

in one culture grow to mature schizonts but become abnormal in the other. Finally a mean D could be given for the average difference between a number of experiments of the same cell type and the controls. This figure is given both in the text and at the foot of each table where growth is assessed by morphology.

The method used in this study proved to be a rapid, reproducible and convenient means of assessing growth of parasites in a culture and the differences between cultures since it incorporated data on all the asexual stages of development and not only the schizonts. However, although the actual score given to each stage correlates roughly with the transverse diameter of the parasite (Fig. 8), the method of assessment is essentially a qualitative one based on morphology. Therefore, the growth scores bear no relationship to the time taken from stage to stage nor the incorporation of isoleucine as a measure of metabolic activity (Fig. 8). For this reason, statistical analysis of the data was not attempted.

Furthermore, the differences (D) should strictly only be compared in experiments where the same donor cells were used, since the time of growth assessment after invasion varied with different samples of donor cells. However, growth was usually assessed at 48 h after subculture and it was the trend of parasite growth in the particular cell type that was of major importance. For these reasons it seems justifiable to further condense the data by the use of a mean difference (mean D) between the controls and test cells of the same type, even though derived from different experiments.

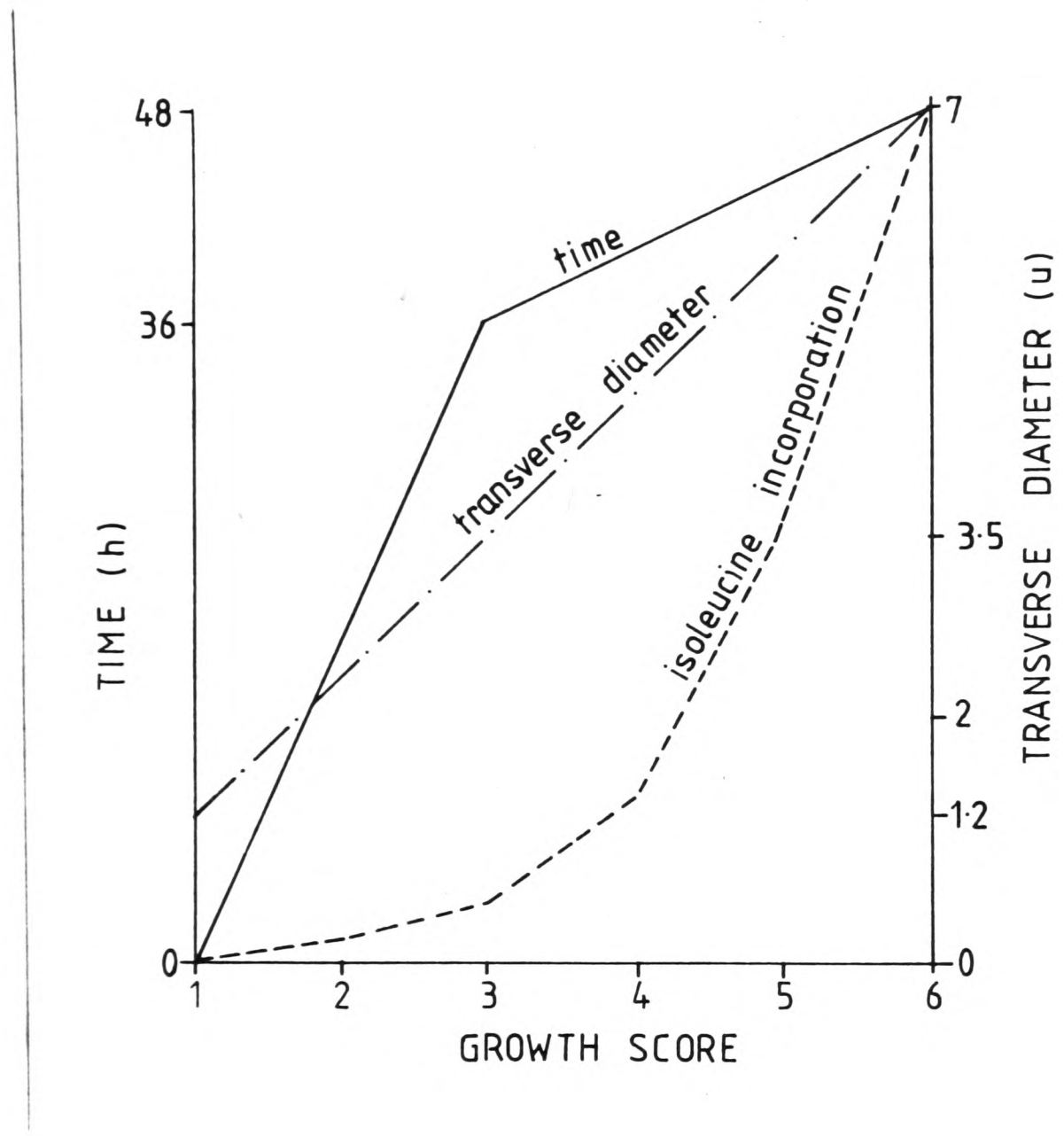


Figure 8 Diagrammatic relationship of time, isoleucine incorporation and parasite diameter to the growth scores allocated to each stage of development of P. falciparum in vitro

(b) Incorporation of tritiated isoleucine

The incorporation of the amino acid isoleucine was also used as a means of assessing parasite growth. At the commencement of a pulse period, 1 µCi of ^{3}H isoleucine was added to each well of the microtissue culture plate which contained the cells to be tested. Each cell type with parasites was tested in triplicate whilst uninfected controls were duplicated. Pulse periods of 12 h were used in order to obtain sufficient incorporation to compare growth of parasites in the various cell types.

At the end of the 12 h pulse period, the contents of each well were mixed and washed into a Durham test tube with PBS. The cells were further washed four times with PBS to remove the unincorporated isoleucine and then transferred in minimal amounts of PBS into glass scintillation vials Two drops (± 100 µl) of hydrogen peroxide (30% w/v) were added to decolourise the haemoglobin and the vials were then left open for about six hours to dry. At the end of this period, 0.4 ml of NCS tissue solubiliser was added to each vial and mixed. After standing for an hour, 6 ml of scintillation solution (Appendix A) was added to each vial. The samples were dark-adapted overnight at room temperature before counting in a Packard Tricarb liquid scintillation counter Model 2002. These counts were corrected for quenching from a quench curve made by plotting percentage efficiency versus external standard counts. Correction for incorporation into non-parasite protein was made by subtracting the mean uptake by uninfected cells from the mean uptake by infected cells.

Where rates of invasion of cell types differed significantly, the mean uptake of isoleucine by the parasites in each well was related to the percentage of viable parasites present at the onset of the pulse period. This excluded any abnormal forms (A) that were present, which were assumed not to be synthesising protein. The ratio between incorporation in the test cells and the control cells (R incorp) is given to compare with incorporation rates in other experiments.

Reproducibility of incorporation results Using the method described above, infected blood was subcultured into microtissue culture plates with normal adult blood and at 12 h intervals wells were pulsed in triplicate with ^{3}H isoleucine. The results from three identical wells were compared (Table 4). The variation of the counts in any triplicate was not greater than 10% from the mean. Throughout this work, incorporation data from infected blood are derived from such triplicate cultures.

Incorporation by uninfected blood In all experiments, incorporation by uninfected cells was gauged from duplicate culture wells. The mean uptake by such cultures subtracted from the mean uptake by infected cells gave the uptake by the parasites in each well. Preliminary experiments were carried out in quadruplicate on two normal adult and two normal cord bloods. Each was tested as whole blood (i.e. including all the white cells), blood from which the buffy coat had been removed after 5 min centrifugation at 600 g, and blood from which white cells had been removed by passing through a column of CF 11 cellulose powder. The results of these control studies are shown in Table 5 and a number of interesting

Expt. No.	Time (h) after subculture	Isoleucine incorporation (dpm) per well				
		(i)	(ii)	(iii)	Mean	Largest % variation from mean
1	19 - 31	15,552	14,803	15,299	15,218	3
	31 - 43	30,913	30,091	32,380	31,128	4
	43 - 55	22,497	19,415	21,279	21,063	8
	55 - 67	14,989	15,426	14,864	15,093	2
2	22 - 34	15,057	14,453	16,997	15,502	10
	34 - 36	24,585	25,870	24,478	25,978	6
	67 - 79	8,718	9,745	8,187	8,883	10
	91 - 103	5,973	6,432	6,312	6,239	4

Table 4 Incorporation of ^{3}H isoleucine by blood infected with P. falciparum to show the reproducibility of the method.

Blood	Treatment of blood	Leucocytes ($\mu l/10^{-3}$)	Isoleucine incorporation (dpm) per well					
			(i)	(ii)	(iii)	(iv)	Mean	Largest % variation from mean
Adult 1 (76144)	Whole blood	2.0	846	962	966	914	922	8
	Buffy coat removed	1.4	629	636	640	680	648	6
	Leucocytes removed by CF 11 powder	0	612	580	630	666	622	7
Adult 2 (76140)	Whole blood	2.5	889	997	945	790	905	13
	Buffy coat removed	1.5	815	728	716	711	743	10
	Leucocytes removed by CF 11 powder	0	576	589	613	550	582	6
Cord 1 (76141)	Whole blood	2.4	2879	2810	2743	2704	2789	3
	Buffy coat removed	1.4	1880	1771	1793	1765	1802	4
	Leucocytes removed by CF 11 powder	0	740	692	745	752	732	5
Cord 2 (76142)	Whole Blood	2.1	1470	1360	1559	1426	1454	7
	Buffy coat removed	1.2	1091	1005	1084	972	1038	6
	Leucocytes removed by CF 11 Powder	0	803	699	776	804	771	9

Table 5 Incorporation of ^{3}H isoleucine by uninfected blood to show the reproducibility of the method.

points arise.

1) Since Hb A contains no isoleucine residues it was thought that there would be a difference in uptake of cord blood with cells containing Hb F (with four isoleucine residues per γ chain) and adult cells, due to the difference in structure of their respective haemoglobins. However, from the Table it can be seen that the large differences are due more to contaminating white cells. Where these cells have been removed, the differences are minimal (mean adult ^{3}H isoleucine incorporation per well 602 dpm, cf cord 752 dpm).

2) The reproducibility of the method for uninfected blood is confirmed. In only one quadruplicate estimation (Adult 2 without leucocute removal) did any count vary by more than 10% from the mean.

3) Although the white cell counts in the adult and cord bloods are comparable, the ^{3}H isoleucine incorporation per white cell is greater in the blood of the newborn.

4) The uptake into adult red cells after cellulose column removal of white cells is presumed to be due to a number of other factors. They are:

i) background counts

ii) non-specific binding of isotope to membranes etc.

iii) labelling of other proteins [illegible] red cell transfer RNA

iv) a few contaminating white cells.

Maximal isotope incorporation by parasites To test whether some factors in the medium might be limiting at high parasitaemias, a preliminary experiment was performed in which increasing percentages of schizont-infected red cells were

subcultured with uninfected cells. The results of the accumulated incorporation of ^{3}H isoleucine over 72 h, measured in triplicate at 12 h intervals are shown graphically in Figure 9. Where the percentage of schizonts was less than 10% there was uptake of isotope up to 72 h. On the other hand at high parasite percentages (22, 37.5 and 50) there was an extremely high uptake in the first 12 h which subsequently diminished. This indicated that at the lower percentages the parasite load was not rate limiting and that the microtissue culture system was adequate to sustain parasite growth for at least 72 h. The percentage schizont-infected cells used in subsequent experiments was thus generally between 2 and 5%.

(c) Photography and weighing of cut-outs of parasites

The third method of assessing parasite growth, carried out by Dr. R.J.M. Wilson, was as follows: Infected Hb A and Hb F-containing cells were distinguished in smears by acid elution. Giemsa stained parasites were photographed on 35 mm film (Recordak 'Microfile') at a magnification of approximately x 530. Infected cells were photographed at random in successive microscopical fields, and, as with morphological assessment, only cells containing a single parasite were selected. Negative images were enlarged 10 times and parasite outlines traced onto graph paper. Relative growth measurements were made from weighed cut-out images.

The reproducibility of this method was tested by photographing four parasites representative of a range of sizes twice each. Enlarged images were drawn from the

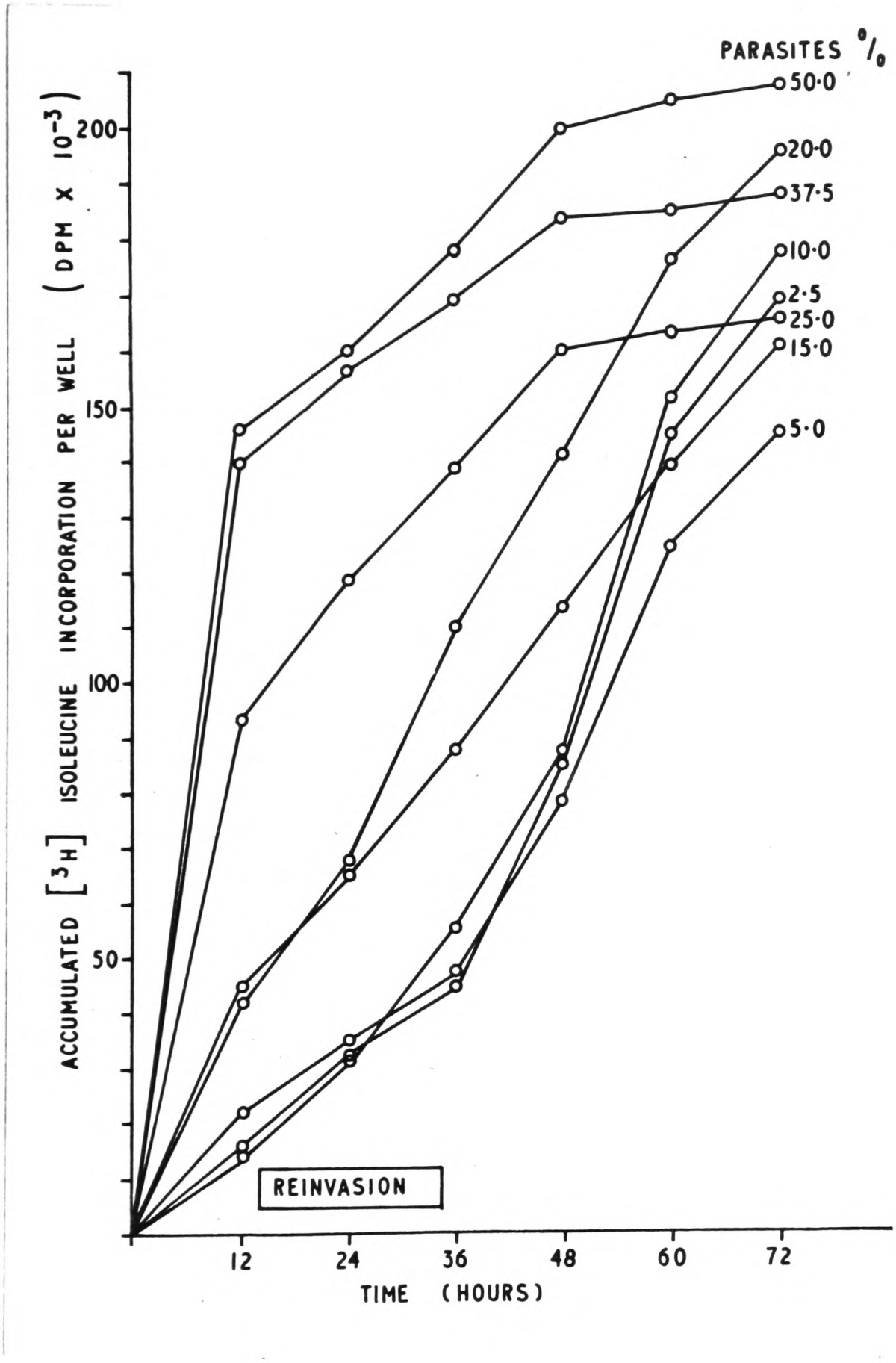


Figure 9 ^{3}H isoleucine incorporation by an increasing percentage of infected cells.

two photographs of each parasite alternately and 31 cut-outs for each parasite were weighed. The results shown in Figure 10 demonstrate the range in estimation of weight (size) of each parasite measured in this fashion.

D. SEPARATION OF RED CELLS BY AGE

Differential centrifugation has been used extensively for the separation of red cells according to age (Borun et al, 1957; Prankerd, 1958; Constandaloukis and Kay, 1959). All these studies indicate that the lighter (more buoyant) cell population represents the younger metabolically active red cells.

In a number of experiments, infected and uninfected red cells were separated on the basis of age by centrifugation. Blood from which the buffy coat had been removed was washed three times with PBS. The packed red cells were then centrifuged in a Wintrobe haematocrit tube at 4°C and 2500 g for 1 h. Wintrobe haematocrit tubes were used in the present study because their length and narrow bore facilitates maximal separation with a relatively small volume (1 ml) of red cells. The column of red cells in the Wintrobe tube which was initially 10 cm long was on average reduced to about 8 cm at the end of this period of centrifugation. Fractions each 0.5 cms long were removed from the top, middle and bottom of the centrifuged cells with a Pasteur pipette. To confirm that this method is capable of separating cells of different ages, blood from a normal adult was treated in this manner, after which reticulocyte counts and assays of glucose-6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase (6PGD)

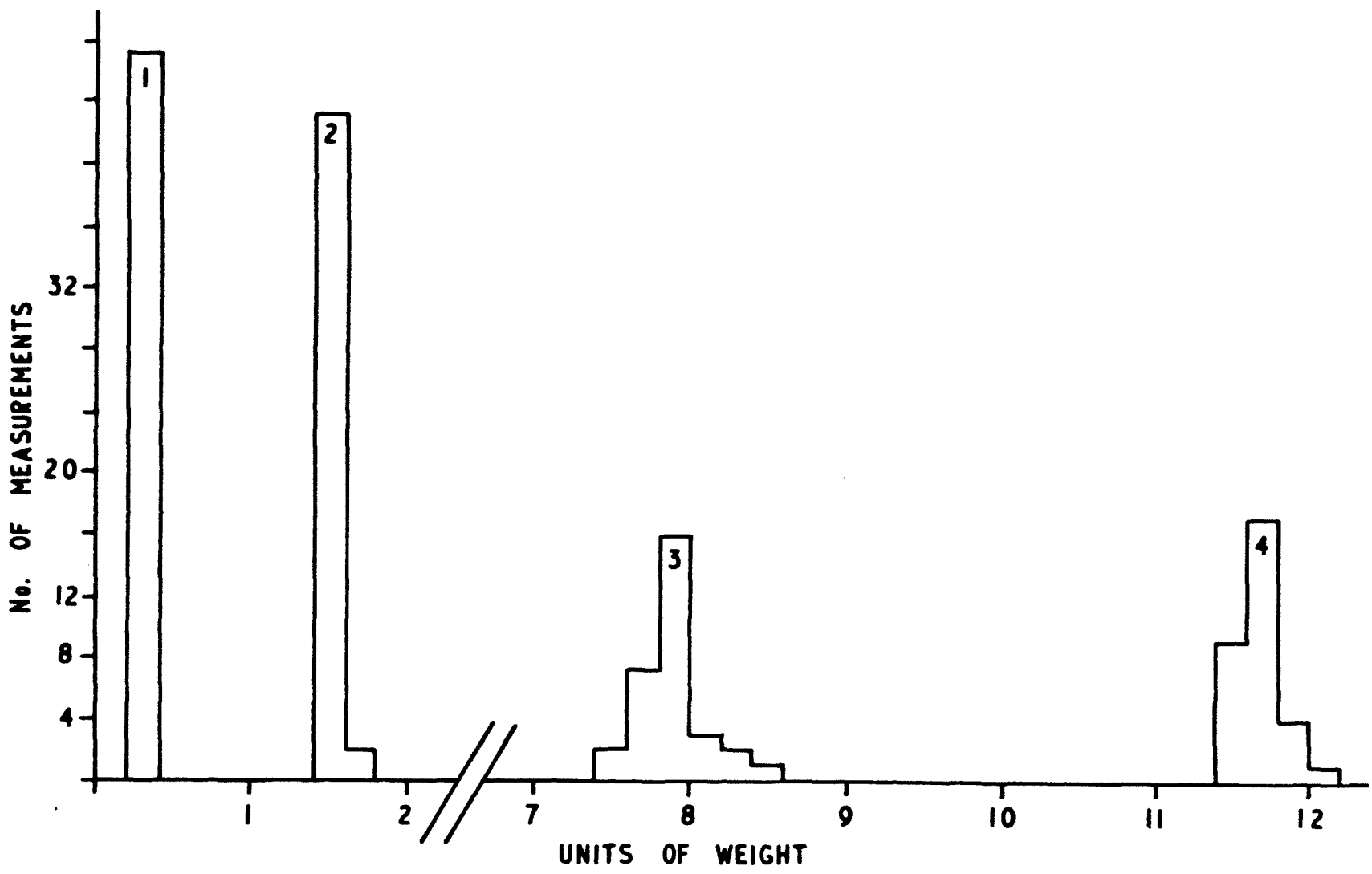


Figure 10 The reproducibility of the method of measuring parasite growth by means of weighing cut-outs of photographed images of parasites.

Parameter	Normal	Cell Fraction				
		Whole blood	Top (T)	Middle	Bottom (B)	T/B
Reticulocytes (%)	< 2	0.8	3.7	0.7	0.3	12.3
G6PD ($eu/10^{10}$ Rbc's)*	1.20-1.72	2.25	3.45	1.98	1.56	2.2
[illegible] ($eu/10^{10}$ Rbc's)	1.00-1.42	1.79	1.96	1.38	1.14	1.7
GOT ($eu/10^{10}$ Rbc's)	0.67-1.07	1.05	1.36	0.95	0.67	2.0

* enzymes units per 10^{10} red blood cells

Table 6 Enzyme activities in different fractions of red blood cells separated on the basis of age by centrifugation.

and red cell glutamate-oxalacetate transaminase (GOT) were performed on each fraction and on whole blood (see Haematological Methods, page 62). These enzyme levels, particularly GOT, are known to correlate well with red cell age (Sass et al, 1964), being highest in young red cells. The results in Table 6 show that the reticulocyte counts and enzyme activities were significantly higher in the top fraction as compared to the bottom fraction with intermediate values for the middle fraction and whole blood. It was concluded that the particular method used in this study to separate cells by age was effective.

E. STAINING METHODS

1. Staining for parasites

Smears were spread, fixed in methanol for 30 seconds and stained with a 5% v/v Giemsa solution diluted with a phosphate buffer (pH 7.2 - 7.4) for 45 min (Appendix A). In later experiments it was found that 20% v/v Giemsa for 5 min gave satisfactory staining.

2. Staining for reticulocytes and parasites

Erythrocytes were incubated with an equal volume of brilliant cresyl blue at 37^{o}C for 10 min. Smears were then made, fixed in methanol and then counterstained with Giemsa's stain.

3. Staining for fetal haemoglobin and parasites

The method of Kleihauer (1957) uses a buffer of low pH (3.3). This elutes Hb A from the red cells. Haemoglobin F is resistant to acid denaturation and hence remains inside the red cell as does the parasite inside an infected cell.

These can then be stained with haematoxylin and eosin as in the original staining method.

It soon became evident in the smears obtained from East Africa using the method of Kleihauer (1957) were not suitable for the visualisation of both parasites and haemoglobin. This method has a number of disadvantages:

(a) Non-visualisation of parasites in cells with large amounts of Hb F, due to the poor staining of the parasite as was demonstrated in Plate I.

(b) Poor morphological detail of the various stages of development of the parasite, particularly with regard to nucleus, cytoplasm and pigment.

(c) Difficulty in visualising erythrocyte membrane in cells from which Hb A had been eluted.

Therefore various methods of staining acid-eluted preparations were attempted (Table 7). It can be seen that there is a distinct underestimation of the percentage of parasites in Hb F-containing cells using haematoxylin and eosin. Diggs et al (1971), who also used Kleihauer's method for acid elution, stained the haemoglobin with eosin and then counterstained with Giemsa (Table 7). This still resulted in fully eluted cells which could be easily missed on microscopy and parasites which stained faintly. This was remedied by using Stevenel's blue, a combination of methylene blue and potassium permanganate (Appendix A) but where this stain was used alone parasite morphology was not clear, especially with regard to nuclear-cytoplasmic differentiation. When Stevenel's blue was used together with Giemsa's stain, there was easy visualisation of parasites in the 'F' cells,

Method of staining	Cell type	No. of cells	No. of parasites	Parasites (%)	Morphology
Giemsa	A + F	659	129	19.6	Clear
Acid elution then:-					
a) Haematoxylin and eosin	A	628	125	19.9	Poor detail of parasites. Fully eluted cell almost invisible.
	F	517	68	13.2	Parasites missed in F cells.
b) Stevenel's blue only	A	655	105	16.0	Poor detail of parasites. Fully eluted cells visible.
	F	661	111	16.8	
C) Giemsa then Stevenel's blue and eosin	A	525	104	19.8	Parasite detail clear. Fully eluted cells visible. Parasites visible in F cells.
	F	526	102	19.4	

Table 7 The effect of various methods of staining on parasite count and morphology in Hb A- and Hb F-containing cells.

clear morphological detail and easy recognition of the cell membrane (Table 7, Plate VII).

The method thus adopted for all subsequent work was as follows: Blood smears were fixed in 80% v/v ethanol for five minutes, washed in tap water and dried. The elution buffer (pH 3.3) was prepared as described in Appendix A. In some cases where the Hb F was heterogeneously distributed amongst the cells, the pH of the buffer was raised to 3.35 with N NaOH. Cells with mixtures of Hb A and Hb F stained bette: after elution at this pH (Plate V). The buffer could be used repeatedly for periods up to a week; this was particularly advantageous for studying cell samples at different times over the period of a single experiment since it maintained consistent elution. After pre-incubating the buffer at 37°C fixed smears were placed in it for five minutes and then washed in tap water. Thus far the method is essentially that of Kleihauer (1957).

The eluted smears were then stained consecutively (without drying) as follows:

(a) Giemsa's stain (5% v/v) for 45 minutes (later 20% v/v for 5 min).
(b) Eosin (1%) for 5 seconds.
(c) Stevenel's blue (Appendix A) for 10 seconds.

The slides were rinsed in tap water and the excess water shaken off after each step.

This modification of staining overcomes the shortcomings of the original Kleihauer method which have been described and allows accurate assessment of invasion and growth of P. falciparum in cells containing Hb F.

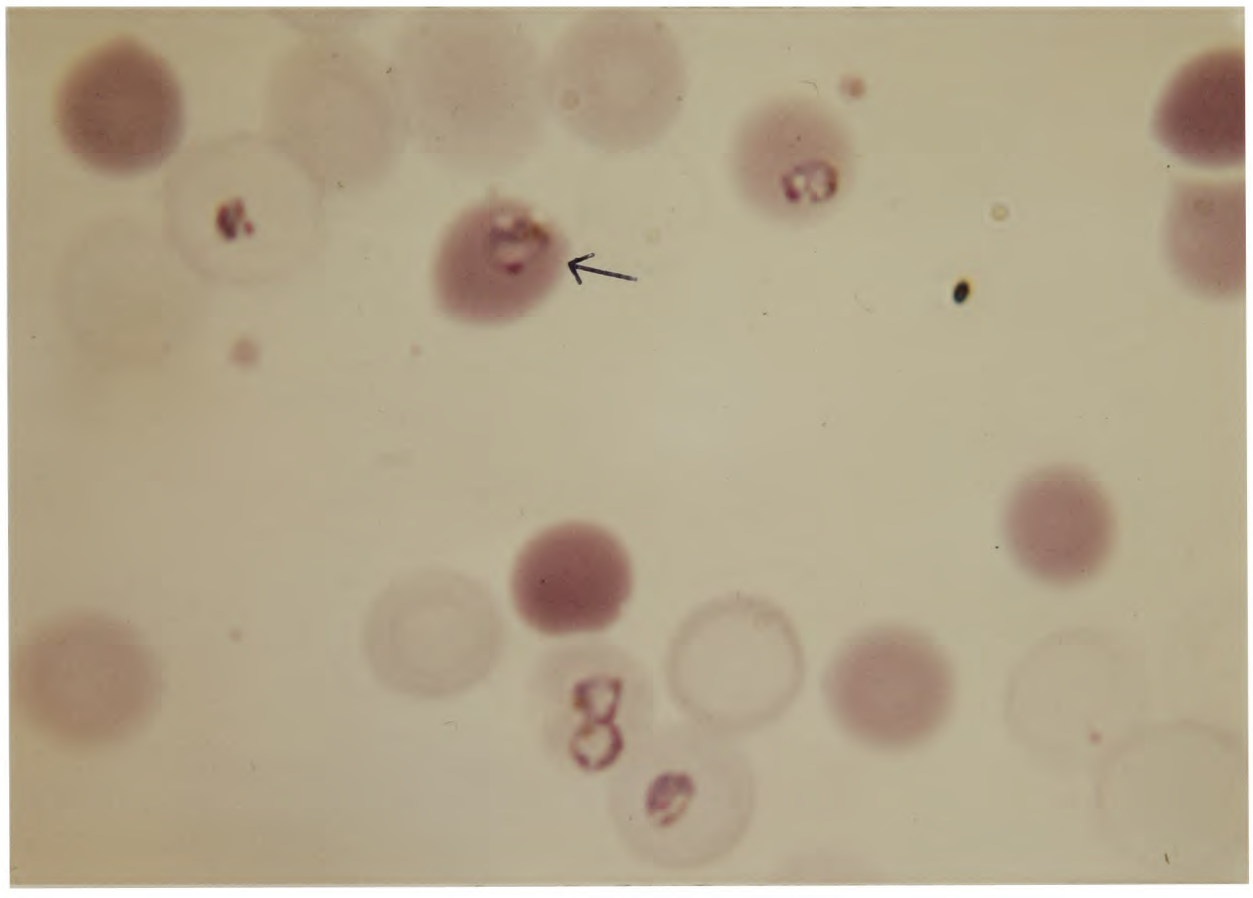


Plate VII Acid elution preparation stained with a modification of the Kleihauer method. Even the parasite in the darkly stained Hb F-containing cell is visible. (x 1500).

F. HAEMATOLOGICAL METHODS

General haematological methods including the sickling test, bilirubin estimations and Field stain method for thick blood films used were those of Dacie and Lewis (1975).

Haemoglobin electrophoresis using starch gel or cellulose acetate strips was done as described by Weatherall and Clegg (1972).

Glucose-6-phosphate dehydrogenase (G6PD) activity was tested in the Gambia using the method of Motulsky and Campbell-Kraut (1961). In the U.K. the quantitative method of Glock and McClean (1953) was used for the estimation of both G6PD and 6 phosphogluconate dehydrogenase (6PGD). Gluconate-oxalacetate transaminase (GOT) activity was assayed via a commercial kit (Boehringer UV system) based on a method by Karmen et al (1955).

Haptoglobin estimation was carried out by a simple radial immunodiffusion method (ICL Scientific).

G. STATISTICAL METHODS

Statistical methods are those as described by Bailey (1959). χ^2 tests were generally used to examine the distribution of parasites amongst various cells of a single experiment. Where data from many experiments was pooled the Student's t-distribution was used.

H. SUMMARY OF METHODS (Fig. 7)

Infected blood was cultured either in flasks until the parasites had grown to the mature schizont stage and then subcultured with the appropriate cells into microtissue culture trays (subculture method) or cultured directly

into these trays after removal of homologous plasma.

Subculture method

1) Samples of 5 ml of blood containing synchronous late ring stages of P. falciparum were collected into heparin (10 iu ml^{-1}).

2) In order to remove white blood cells the blood was layered onto a 6x2 cm column of Whatman CF-11 cellulose in PBS and washed through with 100 ml supplemented Medium 199 with 10 mM HEPES into a 2 L Ehrlenmeyer flask.

3) The flasks were gassed with 5% CO_2 in air and incubated at 37°C with gentle shaking. After approximately 12 h, the cultures were assessed and supplemented with glucose and sodium bicarbonate.

4) To concentrate the schizont-infected cells which were present at about 24 h after initiating the cultures, the red cells were recovered by centrifugation, reconstituted to their original 5 ml volume and mixed with half this volume of Plasmagel (3% w/v gelatin). This mixture was allowed to sediment for 15 minutes at 37°C. At the end of this period two distinct phases were present, with schizont-infected cells in the upper layer. These were retrieved by centrifugation.

5) A suspension of schizont-infected (donor) red cells was made by adding them to a mixture of three parts supplemented Medium 199 and one part non-immune AB serum, allowing 250 µl for each well in the microtissue plate.

6) The uninfected (recipient) cells were collected into

ACD, washed three times with PBS and the buffy coat was removed. These washed cells were added to an appropriate volume of the schizont-infected cell suspension so that the final concentration was approximately $80x10^3$ cells/µl with 2-5% schizont-infected cells.

7) The mixture was plated into the wells of a microtissue culture plate (250 µl per well) and incubated at 37°C under the appropriate conditions.

8) At various times, blood films were made and stained appropriately by standard methods, except that the staining of acid elution preparations was modified to enable easy visualisation of fully eluted cells, parasites and Hb F.

9) Invasion by parasites was assessed by counting at least 1000 cells in each culture shortly after invasion had occurred.

10) Parasite development was assessed by morphology, by the measurement of incorporation of ^3H isoleucine, and by weighing the cut-out images of photographed parasites, after these images had been enlarged.

11) In those experiments where the separation of red cells on an age basis was required, such separation was carried out by centrifuging packed red cells in a Wintrobe tube at 2500 g for 1 h.

CHAPTER 3

BLOOD SPECIMENS: PROCESSING AND DESCRIPTION

NUMBERING OF SPECIMENS

Both infected (donor) and uninfected (recipient) blood samples have been numbered so that the first two figures 74, 75, 76 and 77, denote the year in which the particular experiments were performed. Also, samples were numbered each year from 1 upwards in the order in which they arrived in the laboratories at Fajara. These figures have been included to indicate where certain samples have been used in more than one experiment and in what combinations they have been mixed together.

COLLECTION

All blood taken by venepuncture was anticoagulated with preservative-free heparin (10 iu/ml) except those specimens for G6PD estimation where acid-citrate-dextrose (ACD) was used. Blood taken by fingerprick was collected into heparinised glass capillary tubes (Hawksley & Co.)

TRANSPORT

Since many of the recipient blood specimens were from the United Kingdom or from outlying areas of the Gambia, they were transported by road, rail or air on ice to the laboratories at Fajara. Where possible, a normal adult control blood was included and all specimens used in the same experiment were taken within three hours of each other.

PREPARATION

All recipient bloods were treated in a similar fashion to each other on arrival at the laboratories (Fig. 7). The plasma was removed by centrifugation at 600 g for five minutes, the cells were then washed three times in PBS and the buffy coat was removed by careful pipetting. If not used immediately, usually because suitable parasites were not available, the washed packed cells were suspended in an equal volume of Medium 199 in a sterile stoppered tube and stored.

STORAGE

Storage of uninfected bloods prepared as above was at 4^{o}C for periods of up to a week during which time they were used in experiments only with specimens which had been taken at the same time.

Although Trigg and Shakespeare (1976) have reported a reduction in invasion by P. knowlesi of red cells which have been pre-incubated at 37^{o}C, no data on the invasion and growth of P. falciparum in cells stored at 4^{o}C are available, except that improved growth was seen in cells stored for 21-28 days (Trager and Jensen, 1976). However this might be related to the reduced viability of the leucocytes present after storage. For this reason, an experiment was carried out to see if storage at 4^{o}C under the conditions described above had any effect on the invasion and growth of parasites.

Blood was taken from a normal adult, at three day intervals on four occasions. The experiment was performed on the day of taking the last specimen. An additional

specimen was obtained the day before the experiment. These specimens were each prepared and stored as described above. Each group of stored cells was added to equal aliquots of a schizont-infected red cell suspension. The rates of invasion and subsequent growth as measured morphologically and by isotope incorporation over two 12-hour periods is shown in Table 8. Full morphological growth data are presented here but will be found in Appendix C in subsequent tables. There was no significant difference in the rates of invasion of parasites in the stored cells as compared to that in the control (mean R = 1.0). Subsequent growth, assessed either morphologically (mean D = +0.1) or by ^{3}H isoleucine incorporation differed only slightly in the stored specimens, and certainly showed no correlation with the period of time stored. On this basis blood was used in other experiments after it had been stored for up to a week before use, but it was always tested against control samples which had been taken at the same time.

SOURCE AND SHORT DESCRIPTION OF BLOODS USED

The type and source of blood specimens used are listed in Appendix B. A brief discussion of each is given here to emphasise the attributes of each cell type that was of importance in these studies.

INFECTED (DONOR) CELLS Malarious blood was obtained from patients attending the outpatient clinic at the Medical Research Council Laboratories at Fajara, The Gambia. All patients were routinely screened for malaria by examination of thick blood films stained by the rapid Field method. In this way the most suitable (heavily-infected late ring stage, synchronous P. falciparum infections) could be selected.

Experimental details	Donor cells	7691				
	Recipient cells	76154	76153	76152	76151	76150
	No. of days stored	0 (control)	1	3	6	9
Invasion	Parasites	3.6	3.5	3.5	3.4	3.7
	R	-	1.0	1.0	0.9	1.0
Growth morphology	SR	0	0	0	0	0
	LR	19	20	16	22	20
	ST	31	30	33	34	32
	LT	23	25	22	21	25
	IS	5	6	3	4	3
	MS	1	0	2	3	2
	G	0	0	0	0	0
	A	21	19	24	16	18
	MGS	2.5	2.6	2.5	2.7	2.6
	D	-	+0.1	+0.0	+0.2	+0.1
Isoleucine incorporation a) 1st pulse period (30-42 h)	dpm $x10^{-3}$ per well	6.3	7.6	7.6	7.8	7.3
	R incorp	-	1.2	1.2	1.2	1.2
b) 2nd pulse period (54-66 h)	dpm $x10^{-3}$ per well	7.1	7.8	8.9	6.6	7.6
	R incorp	-	1.1	1.3	0.9	1.1

Invasion: Mean R = 1.0
Growth: Morphology Mean D = +0.1
Isoleucine incorporation Mean R incorp (a) = 1.2
(b) = 1.1

Table 8 The effect of storage of red blood cells at $4^{o}C$ on invasion and growth of P. falciparum in vitro.

After the blood had been collected, appropriate treatment was instituted. The infected donor blood was generally incubated in culture within three hours of venepuncture.

UNINFECTED (RECIPIENT) CELLS 1) Normal adult controls

Each batch of bloods was accompanied by one or more controls. Where it was possible, these were obtained in the United Kingdom from normal adults. Such samples were from individuals who were non-immune to malaria and not on malarial prophylaxis of any kind. In the Gambia, controls were obtained from two sources:-

(a) Healthy Gambian adults who were judged haematologically normal by standard methods such as haemoglobin estimation, absence of parasites on thick blood films, and reticulocyte, white blood cell and differential counts. The sickling test, screening test for G6PD deficiency and haemoglobin electrophoresis were done to exclude any major genetic abnormalities of their red cells.

(b) Healthy Caucasian adults in the Gambia who were on malaria prophylaxis (pyrimethamine 25 mg/week alone or with pyrimethamine 12.5 mg + dapsone 100 mg weekly).

The cells from sources (a) and (b) were washed three times with PBS before use. Comparison of invasion and growth of parasites in control cells obtained either from The Gambia or Caucasians in The Gambia were tested and found to be similar. If the presence of immunity or drug after washing interfered with parasite proliferation, then it would if anything have reduced the differences seen between the controls and those cells which were less susceptible to parasite invasion or growth. Furthermore,

pyrimethamine has been shown to act on only the mature schizont and free merozoite (Gutteridge and Trigg, 1971), presumably because it is unable to penetrate the erythrocyte membrane. Thus, washing the cells should have removed most of the drug. Red cell and serum pyrimethamine and dapsone drug levels were attempted by the Wellcome Research Laboratories, London, but the red cell drug assays were unsuccessful.

2) Cells containing fetal haemoglobin Hb F-containing cells were obtained from five sources - normal newborns, from an individual with $\beta^{o}/\delta\beta^{o}$ thalassaemia, from individuals homozygous for the British form of hereditary persistence of fetal haemoglobin (HPFH) and from a heterozygote for the African form of HPFH, and finally from infants aged 3-6 months. These were chosen because, as will be explained later, the Hb F is contained in cells which are either metabolically younger, older or the same age as the Hb A-containing cells to which they were compared.

a) Hb F cells YOUNGER than Hb A cells

i) Cord cells were collected from the umbilical cord of healthy newborns. These specimens contained about 90-95% cells with detectable Hb F using the modified Kleihauer technique. The cells of newborns are on average younger and metabolically more active than those of normal adults (Oski and Komazawa, 1975).

ii) Thalassaemic blood was collected from a patient described by Ottolenghi et al (1975). This patient, who is a compound $\beta^{o}/\delta\beta^{o}$ thalassaemia heterozygote, has cells which contain almost 100% Hb F with about 1% Hb A_2. He was being venesected (± 450 ml) at regular two-weekly intervals at the time of taking the blood and his reticulocyte count was on average 10%. These cells are metabolically younger

than those of normal adults.

By making an artificial mixture of cord or thalassaemic cells with adult cells it would be possible to compare the rates of invasion of Hb F and Hb A red cell populations with Hb F in the younger cells.

b) Hb F cells of the SAME AGE as Hb A cells

i) Cells from homozygotes for the British form of hereditary persistence of fetal haemoglobin (HPFH) contain Hb F distributed unevenly amongst the cells; some cells have mainly Hb F and others mainly Hb A using the method of Kleihauer (Weatherall et al, 1975). However, more sensitive immunofluorescent techniques have recently shown that all the cells in this condition have at least a small amount of Hb F (Boyer et al, 1977). In three homozygotes, the levels of Hb F ranged from 19.4 - 20.4%. Haematological findings from one of these homozygotes were essentially normal: Hb 13.1 g/dl, RBC 4.86 x 10^{12}/l, PCV 38.1, MCH 36.6 pg, MCV 79 fl, MCHC 34.1 g/dl, reticulocytes 1% and the red cell morphology on a stained film was normal. There was no significant difference between the levels of Hb F in the top or bottom of a sample of such blood centrifuged at 30,000 g for one hour. Morever, haemoglobin synthesis studies showed that the rate of turnover of Hbs F and A were the same in the red cells of these individuals. Hence, the metabolic ages of the Hb A and Hb F-containing cell populations are similar and in such a situation it would be possible to study the relative invasion rates of these cells with different haemoglobins but of the same average metabolic age.

ii) Cells from the African form of hereditary persistence of fetal haemoglobin (HPFH) In this condition, due to a deletion in the γ-δ-β gene complex (Kan et al, 1975) heterozygotes have Hb F distributed evenly amongst all the cells which are otherwise normal, with normal rates of turnover. In 37 heterozygotes, the level of Hb F ranged from 17.3 to 33% with a mean of 26% (Conley et al, 1963). The distribution of Hb F amongst the cells was relatively uniform. The haematological findings including Hb, PCV, MCHC and reticulocyte counts and red cell morphology were normal. Thus these cells are on average of the same age as normal adult red cells.

c) Hb F cells OLDER than Hb A cells

Blood collected from children under the age of about six months contains a variable amount of Hb F unevenly distributed amongst some of the cells. In such infants, Hb F production has almost ceased and the newly-produced red cells contain mainly Hb A (Bratteby and Garby, 1974). Hence, in the same blood sample it is possible to examine the relative rates of invasion of Hb A and Hb F-containing cells. In this case however it is the latter that are the metabolically older population.

3) Cells containing haemoglobin with an increased oxygen affinity

These red cells were obtained from members of a family with polycythaemia due to a haemoglobin variant (Hb Radcliffe). This is due to an amino acid substitution at position 99 of the β globin chain where an alanine residue replaces the usual aspartate. This results in a haemoglobin with an

increased affinity for oxygen ($p50^1$ 12 mmHg - normal p50 27 mmHg). The amino acid substitution at the site of contact of the α and β chains is thought to stabilise the oxyhaemoglobin conformation of the molecule (Weatherall et al, 1977). In these cases the MCH, MCHC, Hb F and reticulocyte counts were normal. Haemoglobin synthesis studies showed that the relative rates of synthesis of B^A and B^{Rad} chains were the same. Hb Radcliffe was therefore turning over in the peripheral blood at the same rate as Hb A indicating that it was not an unstable variant.

4) Sickle cell disorders

Blood from heterozygotes for sickle cell anaemia was obtained from known carriers who were Gambian members of staff of the Medical Research Council at Fajara. Routine haematological examination showed them to be otherwise normal. Haemoglobin electrophoresis confirmed that they were carriers of Hb S. All the cells in this condition contain some Hb S (Papayonnopoulou et al, 1976) although whether this is evenly distributed is not known. In addition, these cells also have a slightly shortened lifespan when compared to normal adult cells.

Homozygous sickle blood was obtained from patients known to have sickle cell disease and who routinely attended the outpatients department at Fajara. They were on daily folate (5 mg) and weekly pyrimethamine (25 mg). By contrast to sickle cell trait, most of the haemoglobin present in these cells is Hb S. There is no Hb A, only varying amounts of Hbs A_2 and F, the latter sometimes reaching levels of 33%

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1 Partial pressure of oxygen required to saturate 50% of the haemoglobin present.

(Milner, 1974) of the total haemoglobin present although this is rare. This Hb F is heterogeneously distributed amongst the older cell population (Gabuzda et al, 1967), unlike the subjects doubly heterozygous for Hb S and the gene for HPFH where it is evenly distributed (Milner, 1974). The red cells in homozygotes also has a markedly shortened lifespan (Milner and Charache, 1973) and thus when compared to normal adult cells, can be considered to be metabolically young.

5) Glucose-6-phosphate dehydrogenase deficiency

G6PD deficient cells were obtained from persons shown to be deficient by screening tests. These individuals came from Keneba, one of the villages in the Gambia surveyed by Dr. I.A. McGregor in 1962. Quantitative assays were carried out on specimens flown to the U.K. to define their G6PD status (i.e. heterozygote or homozygote females, deficient hemizygous males).

G6PD deficiency has a sex linked recessive inheritance (Harris, 1975). Hemizygous males (with only one X chromosome) are thus either normal or deficient. In females, one of the X chromosomes is thought to be activated during early gestation, and thus females heterozygous for G6PD deficiency have some red cells with normal enzyme levels and others which are deficient, depending on which X chromosome is inactivated. This mixture of normal and deficient cells is known as mosaicism. Since the inactivation of the X chromosome is a random process, the overall enzyme level in the blood of such a heterozygote can vary from nearly normal to almost totally deficient, although the majority will have levels close to 50% of normal with an equivalent number of non-deficient

cells. Homozygous females, like deficient hemizygous males, will be totally deficient.

Whereas young red cells in this condition have normal enzyme activity, older cells are grossly deficient in G6PD and are abnormally sensitive to oxidants. However, in the absence of these oxidants, red cell survival in deficient individuals is only slightly less than normal (Bernini et al, 1964; Brewer et al, 1961).

6) β Thalassaemia trait

These were obtained from known carriers living in the Oxford area in England. Cells from such individuals are both small in size and contain small amounts of haemoglobin when compared to normal adult cells. They also have a marginally higher rate of turnover than normal cells (see Weatherall and Clegg, 1972).

7) Iron deficiency

The hypochromic microcytic cells were obtained from a patient admitted to the Radcliffe Infirmary, Oxford with chronic bleeding from a peptic ulcer. The MCH was 20 pg. In this condition the red cells have only a minimally shortened lifespan (Loria et al, 1967).

8) Haemoglobin C and E trait

These cells were obtained from patients living in London. There are only minor haematological abnormalities in these conditions and the red blood cell lifespan is normal (Bunn et al, 1977). Target cells are the main morphological feature in Hb C trait.

CHAPTER 4

PARASITE INVASION

A PRELIMINARY STUDY OF THE DISTRIBUTION OF P. FALCIPARUM IN RED CELLS CONTAINING HAEMOGLOBIN A OR F.

On at least theoretical grounds, the protective effect of Hb F against falciparum malaria seemed to be a reasonable working hypothesis. Therefore, a series of pilot experiments was undertaken to ascertain whether a more detailed examination of the problem would be worthwhile. Preliminary studies were undertaken to see if there was any difference in the cellular distribution of P. falciparum in blood taken from individuals with malaria and with red cells containing varying amounts of Hb F and Hb A. Such a situation occurs in vivo in young infants infected with P. falciparum and who are under about six months of age when both haemoglobins are present. However, since falciparum malaria is relatively infrequent and asymptomatic in this age group (Bruce Chwatt, 1952), it was necessary to screen many individuals to find appropriate cases.

For screening purposes, thick blood films were made from samples obtained by fingerprick from infants aged three to six months and collected into heparinised glass capillary tubes (Hawksley & Co.) After air drying, these films were stained by the rapid Field method. Thin smears made from samples which contained parasites were stained either with Giemsa or by a modification of the acid elution technique of Kleihauer. The percentage parasitisation of the cells

was determined using both the Giemsa stain and the acid elution technique (Table 9). The good agreement of parasite counts between the two methods confirmed that the modified elution procedure did not result in loss of ring forms, and that the modified method of staining these preparations did not obscure the parasites in the deeply-stained Hb F-containing cells. The relative distribution of parasites in the various cell types, cells containing mainly Hb A (A), cells containing both Hbs A and F (I - intermediate) and cells containing mainly Hb F (F), was determined by counting at least 2000 cells in each case (Table 9). In eighteen samples examined, there was a relative paucity of parasites in the fetal and intermediate cell populations. Using the χ^2 test these differences were significant ($p < 0.01$) in thirteen cases and not significant in five cases ($p > 0.10$) (Fig. 11). A typical acid elution preparation from which these data were obtained is shown in Plate VIII.

In order to confirm these findings, the slides were examined using a microdensitometer (Shepard et al, 1962). To see whether the visual classification into adult, intermediate and fetal cells was valid, the cells were assigned to one of these groups before their optical density (OD) was determined. Although there was some overlap between the groups the visual classification proved remarkably good (Fig. 12). The ODs of the parasite-containing cells were then compared with those of the total cell population. The results of a typical experiment are shown in the same figure which indicates that the parasites are found in the cells of lower OD, i.e. those containing predominantly Hb A. The slight increase in the OD of the parasitised cells as

Expt. No.	Red blood cell type (%)			Parasites (%)					
	Acid elution			Giemsa	Acid elution				
	A	I	F	All Cells		A	I	F	A/F
1*	70	16	14	4.6	4.3	4.5	3.7	4.2	1.1
2*	89	7	4	17.4	16.5	16.4	18.9	12.7	1.3
3*	43	19	38	1.6	2.0	2.3	2.0	1.5	1.5
4*	65	29	6	13.0	13.0	13.1	13.3	7.6	1.7
5*	51	21	28	2.0	2.6	3.7	1.6	1.5	2.5
6	79	18	3	4.8	5.2	5.8	3.2	1.8	3.2
7	49	19	32	10.6	11.7	17.1	9.1	4.9	3.5
8	38	54	8	2.0	2.0	2.0	2.1	0.5	4.0
9	80	15	5	2.0	2.0	2.3	0.8	0.5	4.6
10	78	16	6	3.4	3.8	4.0	2.8	0.7	5.7
11	87	6	7	15.1	16.1	17.8	6.4	2.9	6.1
12	53	14	33	3.2	2.5	3.9	2.1	0.6	6.5
13	46	30	24	6.3	7.2	12.1	4.0	1.7	7.1
14	73	14	13	4.8	3.8	4.6	2.8	0.6	7.7
15	36	44	20	9.0	7.0	9.5	7.8	1.1	8.6
16	75	10	15	4.0	4.1	5.0	3.2	0.5	10.0
17	71	21	8	4.8	4.5	5.6	2.2	0.5	11.2
18	28	38	35	3.2	3.7	6.3	5.1	0.4	15.8

* Difference in distribution of parasites not statistically significant $p > 0.1$

Table 9 Distribution of P. falciparum between the Hb A-containing cells (A), cells containing both Hb A and Hb F (I-intermediate) and cells containing mainly Hb F (F) in blood from infected infants.

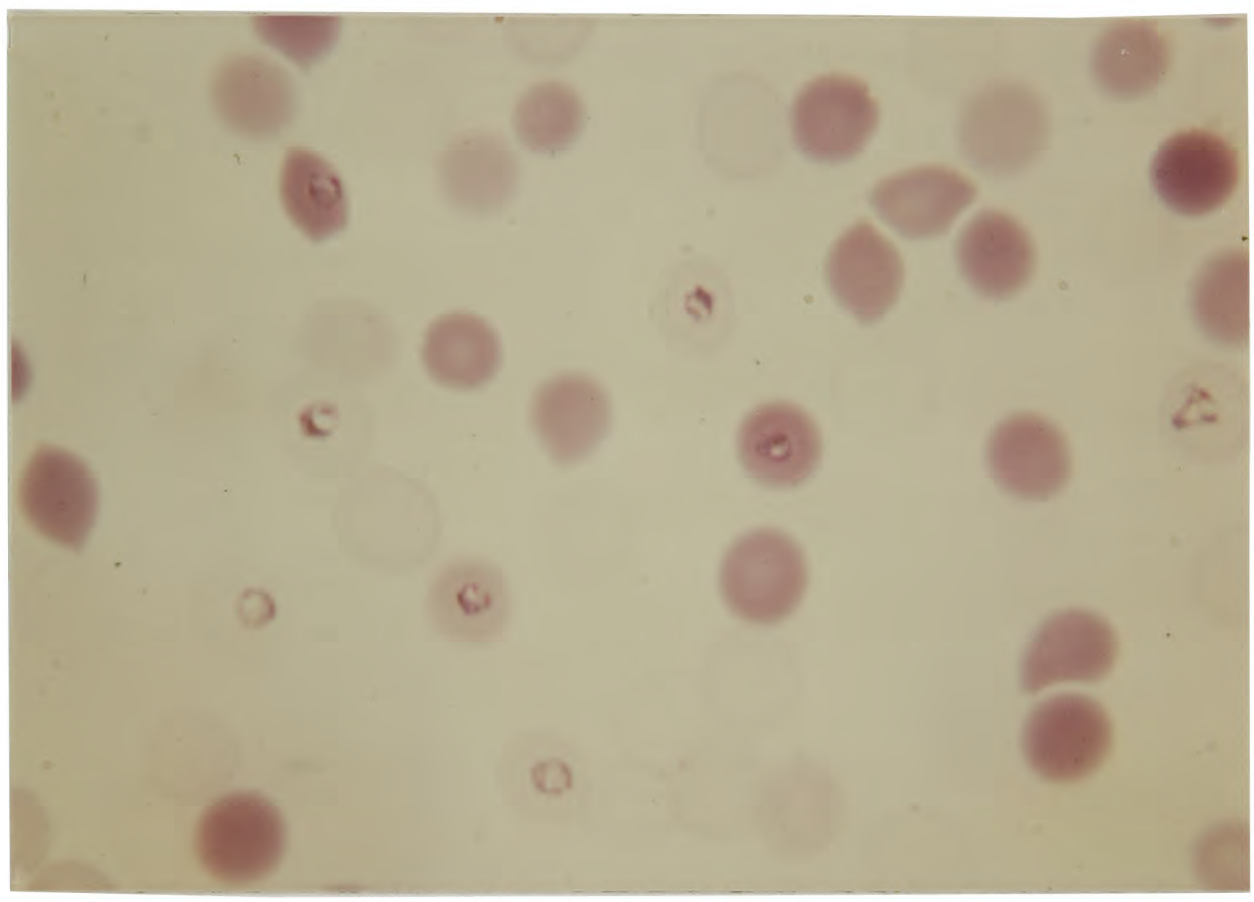


PLATE VIII Typical acid elution preparation from an infant infected with P. falciparum. Note the presence of parasites in the fully eluted cells.

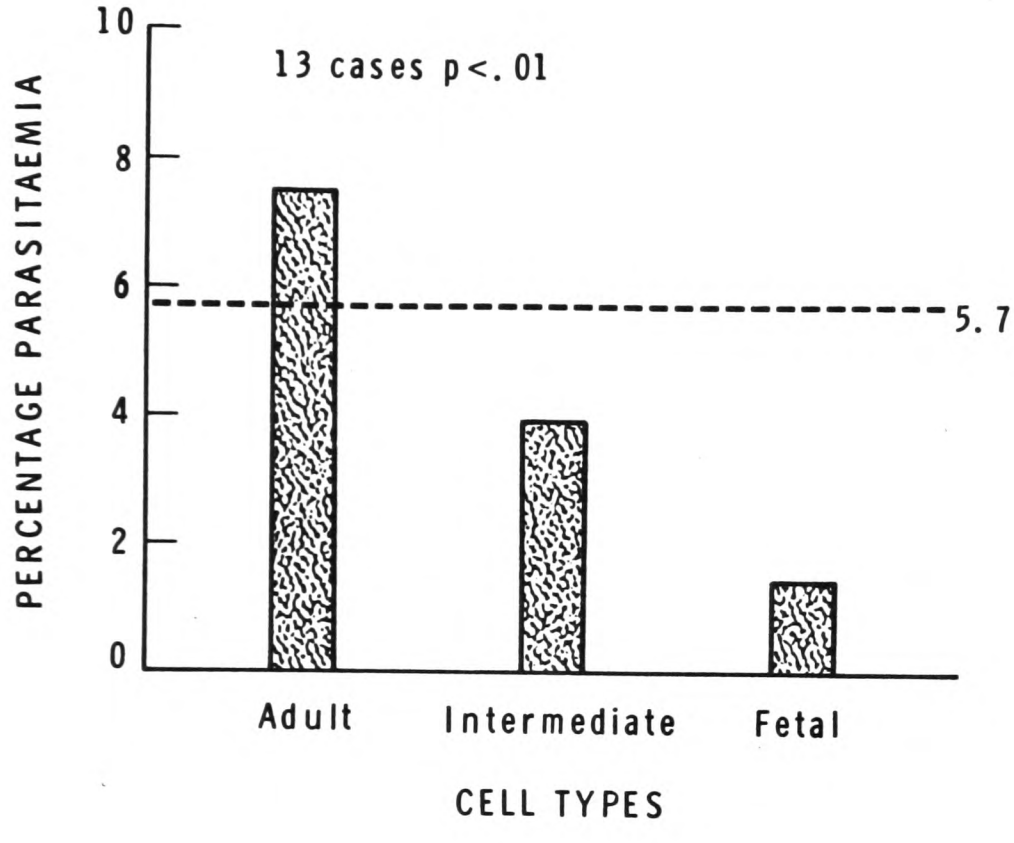


Figure 11 The distribution of P. falciparum in vivo between Hb A-containing cells (adult), cells containing Hb A and Hb F (intermediate) and cells containing mainly Hb F (fetal).

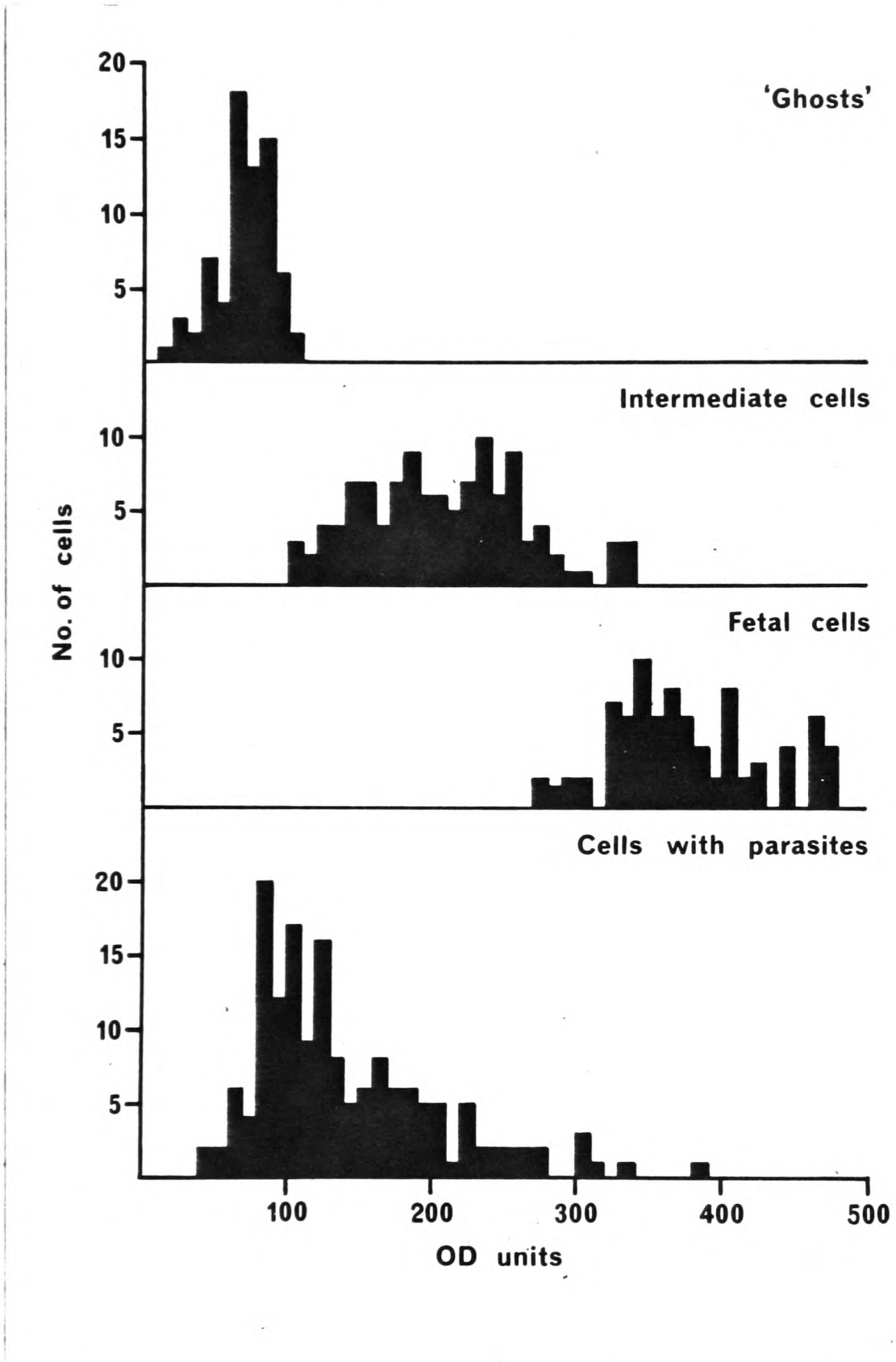


Figure 12 Acid elution preparation from the blood of a three-month-old infant with P. falciparum malaria studied by microdensitometry.

compared with those of the fully eluted cells may partly result from an increase in OD due to the presence of the parasite. This preliminary study showed a significant difference in parasite distribution between Hb F, and Hb A-containing cells in the blood of naturally infected infants. In most of the experiments, there was a paucity of parasites in the Hb F population and this was not due to an elution or staining artefact. These results were encouraging and certainly indicated that the problem was worth further study. In particular they raised the question of why the parasites were more frequently found in Hb A-containing cells.

EXPLANATION OF THE UNEVEN DISTRIBUTION OF FALCIPARUM PARASITES AMONGST Hb A AND Hb F-CONTAINING CELLS

Further experiments were directed at elucidating a possible mechanism to explain the paucity of parasites amongst the Hb F-containing cells. Firstly, since Hb F is replaced by Hb A during the first year of life, the Hb F-containing cells in young infants represent an older population, and thus the parasite could have a preference for the relatively younger Hb A-containing red cells in such a situation. Secondly, the Hb F itself could be a deterrent to parasite invasion, whilst a third possibility could be the preferential removal of the parasitised Hb F-containing cells. A fourth, but unlikely explanation could be the failure of development of a parasite once inside a Hb F-containing cell. This would imply that such a parasite could not be visualised, which seemed unlikely.

The experiments described below were designed to distinguish between the first three of these explanations.

A CELL AGE AND INVASION

1) Distribution of P. falciparum in reticulocytes and non-reticulocytes

Red cells from patients with severe falciparum malaria (greater than 100 parasites per high power field on thick blood films) were incubated with brilliant cresyl blue for reticulocytes, smeared and then counterstained with Giemsa stain for parasites. Counts were done on cases where the parasite was in the small ring stage. This was to avoid the possibility that a cell, which when invaded by a parasite as a reticulocyte, had lost its reticulum at the time the counts were done. The relative rates of invasion of reticulocytes and non-reticulocytes were assessed by counting the number of parasites per thousand cells of each type and are shown in Table 10. The percentage parasitaemia ranged from 1.7 to 13.1% and in each case a greater proportion of reticulocytes was parasitised than non-staining reticulocytes. Using the χ^2 test, this difference was found to be significant at the 1% level in all cases.

2) Separation of infected red cells on the basis of age by means of centrifugation

Parasite counts in cells infected *in vivo* and then separated according to age by centrifugation are shown in Table 11. These include both small and large ring stages. Evidence of effective separation is provided by the higher reticulocyte count in the top fraction and the virtual absence of reticulocytes at the bottom. In all ten cases examined there was a greater number of ring forms in the top fraction

Expt. No.	Blood	Reticulocytes (%)	Parasites (%)	Parasites in 1000 reticulocytes (r)	Parasites in 1000 non-reticulocytes (nr)	r/nr
1	75119	8.0	8.1	114	72	1.6
2	76122	10.0	1.7	17	10	1.7
3	7675	2.7	2.2	47	24	2.0
4	75206	1.3	13.1	220	110	2.0
5	76134	2.0	7.4	180	80	2.3
6	76117	4.2	5.8	165	64	2.6
7	75118	3.5	2.2	42	15	2.8
8	75117	1.5	3.1	102	25	4.1

Table 10 Distribution of P. falciparum in reticulocytes and non-reticulocytes in the blood of eight infected patients.

of young cells as compared to the middle or bottom fractions. These were significant at the 1% level using χ^2 tests for each sample.

It was considered possible that the ring forms of the parasite might alter the buoyancy of the cell, as occurs with the later forms of P. falciparum (Geiman et al, 1946). Therefore 100 infected cells were counted in each fraction and the percentage of multiply infected cells noted. The rationale of this was that if a ring form of the parasite were to make the red cell more buoyant, then this effect would be even more pronounced in a cell containing more than one parasite. If this were so, it would be expected that a large proportion of these multiply infected cells would be found in the upper fractions of the centrifuged samples. However, multiply infected cells were fairly evenly distributed throughout all the fractions (Table 11), indicating that it was unlikely that the ring forms of the parasite had significantly altered cell buoyancy. This was confirmed by the experiment where gelatin concentrated the more mature forms of the parasite but not the ring forms (page 50).

3) Centrifugation of uninfected cells prior to infection with P. falciparum

To examine further the relationship of red cell age to parasite invasion, avoiding any possible alteration of buoyancy of red cells by the parasite, uninfected blood from healthy donors was centrifuged as before (i.e. in Wintrobe haematocrit tube at 2,500 g for 1 h at $4^{o}C$) to separate them into age groups prior to infection with donor cells. Cells from whole blood, the top, middle and bottom

Expt No.	Reticulocytes (%)				Multiply infected cells (%)*				Parasites (%)				
	Whole blood	Top	Middle	Bottom	Whole blood	Top	Middle	Bottom	Whole blood	Top (T)	Middle	Bottom (B)	T/B
1	1.5	4.7	0.8	0.4	1	2	2	3	2.2	4.3	2.6	2.5	1.7
2	-	6.9	5.1	1.9	0	0	2	2	3.4	3.9	1.9	1.2	1.7
3	-	0.6	0.2	0.2	17	16	13	16	31.0	35.9	24.1	20.6	1.7
4	-	5.8	2.9	1.3	5	5	2	3	3.5	5.6	3.0	2.1	2.7
5	0.3	5.7	0.2	0	10	19	16	18	11.5	30.6	12.8	11.4	2.7
6	-	4.6	0.7	0.1	9	8	7	11	3.4	6.1	3.8	1.9	3.2
7	8.9	19.9	5.6	1.2	6	5	4	3	7.5	17.3	6.7	5.0	3.5
8	-	7.6	1.8	0	11	9	7	8	8.6	17.0	9.3	3.5	4.9
9	5.0	74.8+	2.8	0.5	2	2	1	0	2.2	4.9	1.3	0.6	8.2
10	1.9	4.1	0.7	0.1	10	6	11	11	13.9	30.1	13.5	3.2	9.2

* expressed to the closest whole percentage of infected cells (i.e. not all cells)

\+ confirmed

Table 11 Distribution of parasites in red cells infected by P. falciparum in vivo and then separated on the basis of age by centrifugation

fractions of the centrifuged specimen were subcultured with an inoculum of schizont-infected red cells in microtissue culture trays. The percentage of small ring stage parasites in each fraction following reinvasion in vitro is shown in Table 12. In each case, there was at least a twofold greater rate of invasion in the younger cells taken from the top of the centrifuged specimen than in the older cell population at the bottom. Cells from whole blood and the middle of the centrifuged sample occupied an intermediate position with regard to parasitisation. Using the χ^2 test, these were all statistically significant ($p < 0.01$). In one experiment (Expt. No. 5), the uninfected cells were cord cells and by means of the acid elution technique only parasites in the Hb F cells were counted, thus omitting invasion of uninfected cells from the donor blood which are inevitably present to some degree. There was no appreciable difference, indicating that infection of uninfected donor cells was not altering the results.

B HAEMOGLOBIN F AND INVASION

In order to examine whether Hb F had any effect on parasite invasion in vitro, uninfected cells containing Hb F in cells of all metabolic ages were subcultured with inocula of schizont-infected cells. These Hb F-containing cells were described in Chapter 3 and Appendix B. Cells which had been taken from infected infants were placed directly into culture in microtissue culture trays. Where mixtures of Hb A-and Hb F-containing cells were placed in the same culture wells, they could be later distinguished from each other by the acid elution technique. However, the African HPFH and

Expt No.	Reticulocytes (%)				Parasites (%)				
	Whole blood	Top	Middle	Bottom	Whole blood	Top (T)	Middle	Bottom (B)	T/B
1	-	-	-	-	2.8	4.3	3.0	2.2	2.0
2	-	-	-	-	3.0	4.3	2.4	2.0	2.2
3	0.4	2.5	0.6	0.2	5.0	7.9	3.6	3.3	2.4
4	2.4	9.6	1.5	0.1	5.1	8.9	4.5	3.2	2.8
5	-	-	-	-	2.3	2.9	1.7	1.0	2.9

Table 12 Invasion by P. falciparum of red cells separated on the basis of age by centrifugation prior to infection in vitro.

some cord cell samples were grown in parallel cultures to the adult control cells, and not mixed together in the same well. In the case of the African HPFH this was necessary because these cells, containing about 25% Hb F, are often difficult to distinguish from Hb A-containing cells on an acid elution smear and in particular when parasitised.

A few cord cell experiments were also carried out in parallel to ensure that parasitisation itself did not cause elution of, or obscure the Hb F. This was of particular importance when dealing with the larger and more mature parasites in the assessment of parasite development. Growing cultures in parallel obviated the use of the acid elution technique and both adult and cord cells could be stained by the Giemsa method which gives the best morphological detail.

The distribution of parasites in cells containing mainly Hb A (A), Hb F (F), or both (I - intermediate - if these were present) was assessed after invasion had been completed by counting at least 2000 cells in each experiment. The results of these studies are summarised in Tables 13a and b, 14 and 15. In the experiments with cord or thalassaemic cells and adult red cells, the Hb F-containing cells were preferentially invaded (Table 13a, Plate IX). Closer analysis of two of the cord and adult cell mixtures in experiments 3 and 4 (Table 13b) shows that, although the increased parasitisation of cord cells was in part due to multiple infection of these cells (Plates X and XI), almost twice as many cord cells were infected compared to the adult controls. Throughout this study, it was found that there

Cells tested	Expt. No.	Donor cells	Recipient cells	Cell type (%)		Parasites (%)		
				A	F	A	F	F/A
Cord and adult cells in the same culture	1	7553	A 7553 / F 758	39	61	5.5	9.9	1.8
	2	7545	A 7545 / F 7533	24	76	9.7	19.4	2.0
	3	742	A 742 / F 741	54	46	5.2	12.1	2.3
	4	743	A 743 / F 741	63	37	5.1	13.2	2.6
Cord and adult cells in parallel cultures	1	7662	A 7660 / F 7659	Equal numbers of cells in each parallel culture		6.1	8.7	1.4
	2	7686	A 7683 / F 7685			16.8	23.0	1.4
	3	76112	A 76100 / F 76105			6.2	10.1	1.6
	4	764	A 7610 / F 7611			7.8	12.1	1.6
	5	76115	A 76100 / F 76105			3.1	5.1	1.7
	6	7661	A 7653 / F 7651			7.9	19.6	2.5
$\beta^{o}/\delta\beta^{o}$ thalassaemia and adult cells in the same culture	1	75110	A 7525 / F 7527	40	60	3.3	5.0	1.5
	2	75111	A 7525 / F 7527	42	58	1.9	7.1	3.7

Table 13a Invasion by P. falciparum of Hb F-containing cells metabolically YOUNGER than the Hb A-containing cells in the cultures

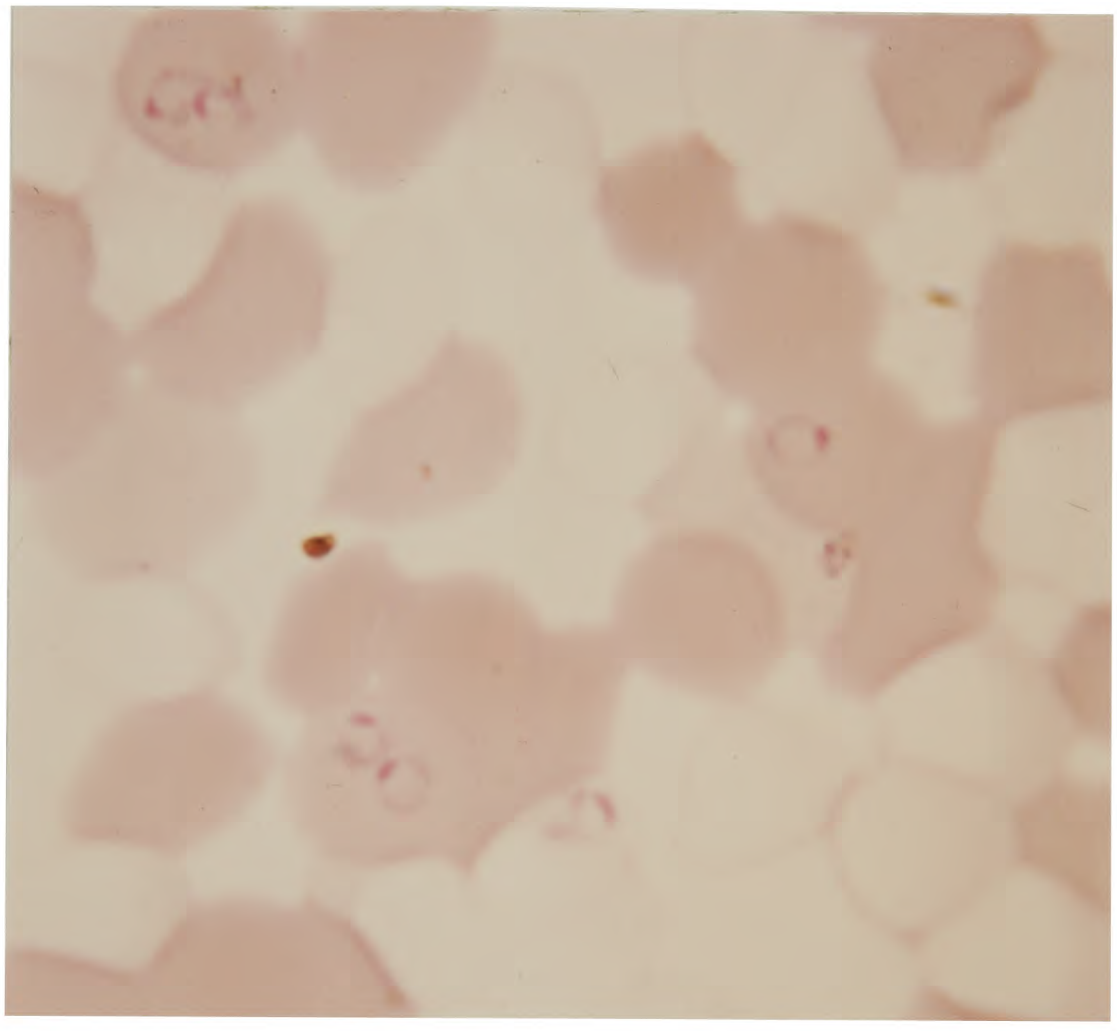


PLATE IX Distribution of P. falciparum in cells containing Hb F which are YOUNGER than those containing Hb A (cord and adult x 1700).

Expt. No (from Table 13a)	3			4		
Cell type	A	F	F/A	A	F	F/A
Number of cells examined for second generation parasites	1183	990	0.8	703	409	0.6
Parasites (%) (parasites/100 cells)	5.2	12.1	2.3	5.1	13.2	2.6
Cells parasitised (%) (parasitised cells/100 cells	4.1	7.4	1.8	3.1	6.9	2.2
Mean no. of parasites/ infected cells	1.3	1.6	1.2	1.6	1.9	1.2
Mean no. of parasites/ multiply infected cells	2.4	2.5	1.1	2.8	2.9	1.0

Table 13b Invasion by P. falciparum of Hb F-containing cells showing the effect of multiple invasion on parasite counts.

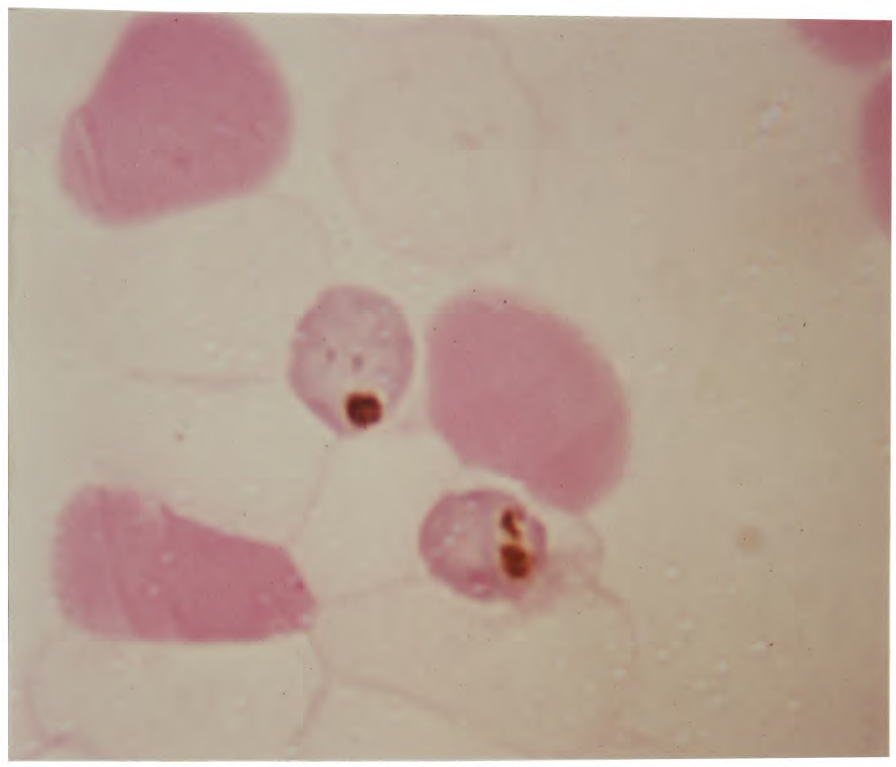


PLATE X Culture of P. falciparum in a mixture of cord and adult cells. Note the large trophozoites present in only the Hb A-containing cells (x 1500).

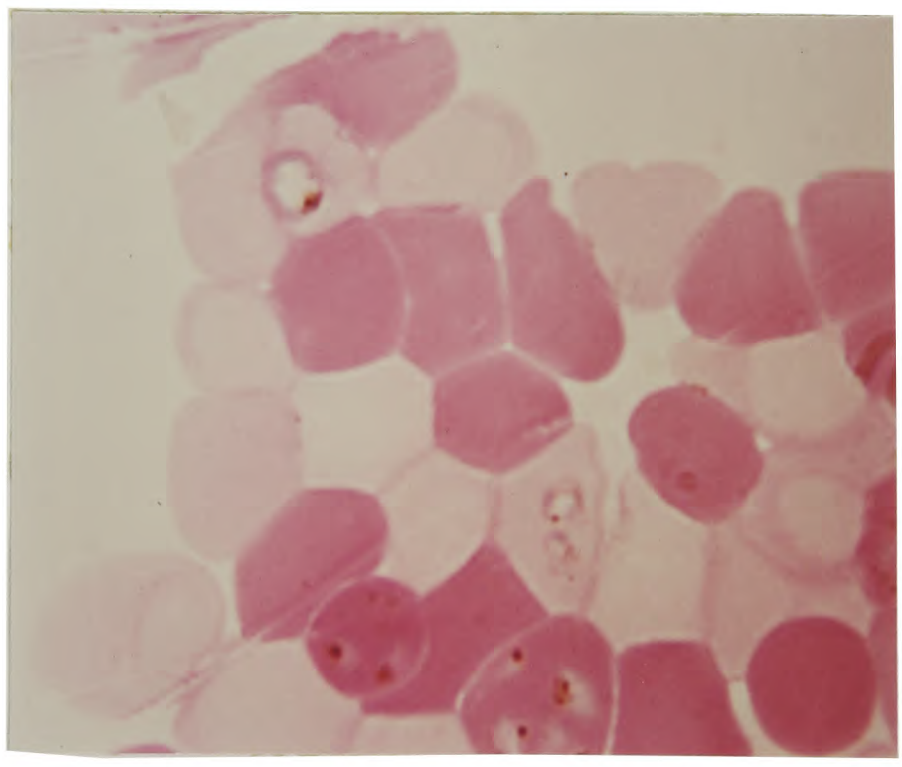


PLATE XI Note the small trophozoites in both cell types but more cord cells are infected and they are more often multiply infected (x 1500).

were slightly more multiply infected cells found on average in younger cells whether they were in the same or parallel cultures.

In the cells of the British and African HPFH (Table 14), the Hb F-containing cells were equally invaded when compared to normal adult controls (Plate XII), whilst in the cells of young infants there were fewer parasites in the Hb F-containing cells in five out of six cases (Table 15, Fig. 13, Plate XIII).

Statistical methods revealed that where Hb F-containing cells were either younger or older than the Hb A-containing control group these differences were significant using the χ^2 test ($p < 0.01$). In the cells of similar metabolic age (British and African HPFH) there was no significant difference in the rates of invasion of cells with either haemoglobin ($p > 0.10$).

The only two exceptions found were those marked with an asterisk in the infant group (Table 15). It is interesting to note that these two cases were amongst the five in Table 9 where the distribution of parasites was not significantly different in the original samples taken from these infected infants. A possible explanation for this is that Hb F production was prolonged in these infants so that Hb F-and Hb A-containing cell populations were of a similar metabolic age. Alternatively, these cells might have been infected by a genetic variant of the parasite capable of equal invasion of both young and old cells. Such variants are known to exist for P. berghei (Walliker et al, 1976). Thus it can be seen that where cells containing Hb F were younger,

Cells tested	Expt. No.	Donor cells	Recipient cells	Cell type (%)			Parasites (%)			
				A	I	F	A	I	F	F/A
British HPFH cells	1	7546	7544	54	33	13	10.8	8.9	10.1	0.9
	2	76112	76104	56	21	23	8.5	8.5	8.4	1.0
	3	7549	7544	57	27	16	4.6	4.3	4.9	1.1
	4	7613	763	42	23	35	8.7	6.9	9.9	1.1
	5	7613	762	41	19	40	9.2	8.5	9.8	1.1
	6	7545	7544	61	21	17	9.6	9.0	10.2	1.1
African HPFH and adult cells in parallel culture	1	76113	A 76100 / F 76109	Equal numbers of cells in each parallel culture			7.8	-	6.7	0.9
	2	76115	A 7660 / F 76109				3.1	-	3.0	1.0
	3	76112	A 76100 / F 76109				6.2	-	6.1	1.0

Table 14 Invasion by P. falciparum of Hb F-containing cells metabolically of the SAME AGE as the Hb A-containing cells in the cultures.

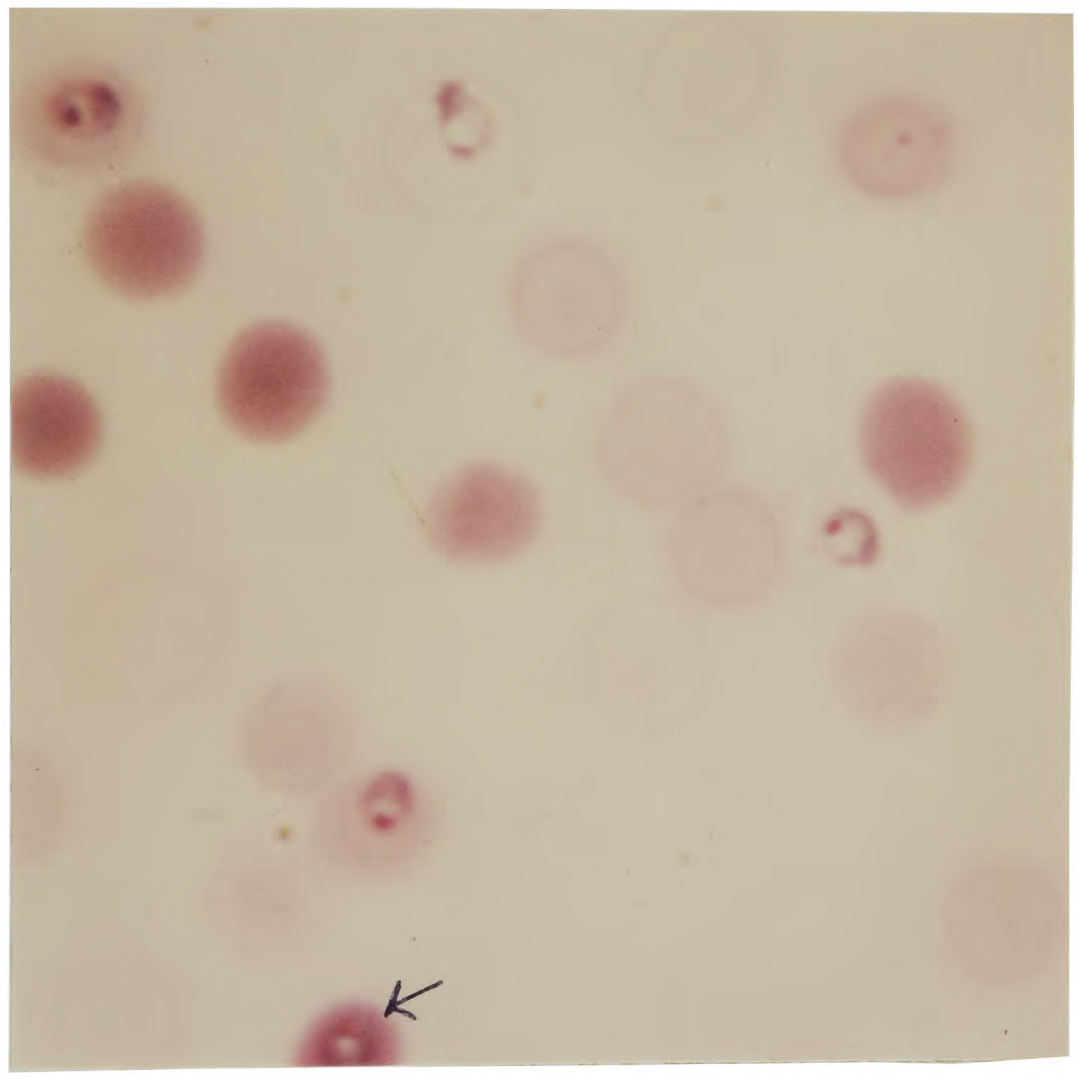


PLATE XII Distribution of P. falciparum in cells containing Hb F which are the SAME AGE as those containing Hb A (British HPFH) (x 1700). The arrow indicates a parasite in a deeply-stained Hb F-containing cell.

Cells tested	Expt. No.	No. of expt. from Table 9	Cell type (%)			Parasites (%)			
			A	I	F	A	I	F	F/A
Infected infant cells (2nd generation)	1	19	31	15	54	4.3	1.6	0.9	0.2
	2	14	66	16	18	7.8	4.0	1.9	0.2
	3	16	72	13	15	7.4	2.9	2.4	0.3
	4	5	65	20	15	12.2	7.0	5.8	0.5
	5	3	55	17	28	3.6	3.6	1.4	0.7*
	6	1	56	24	20	2.9	1.2	3.2	1.1+

* $0.02 > p > 0.01$

\+ not significant $p > 0.10$

Table 15 Invasion by P. falciparum of Hb F-containing cells metabolically OLDER than the Hb A-containing cells in the cultures.

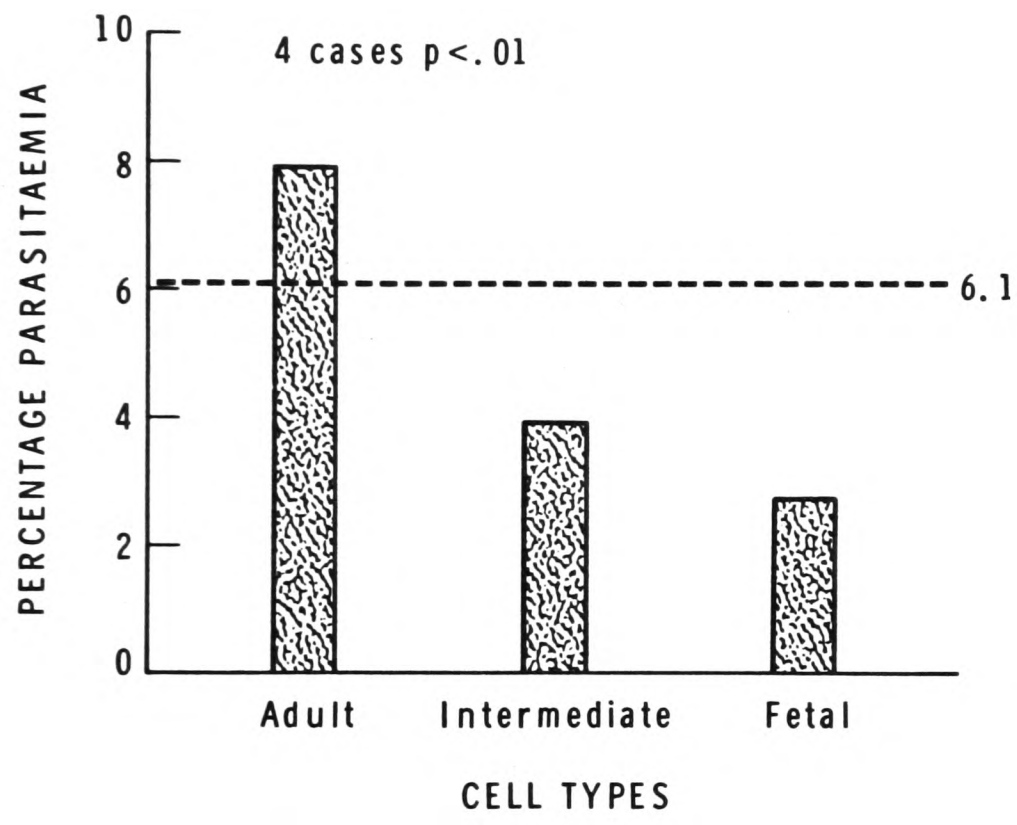


Figure 13 Invasion by P. falciparum of Hb F-containing cells metabolically older than the Hb F-containing cells in the cultures.

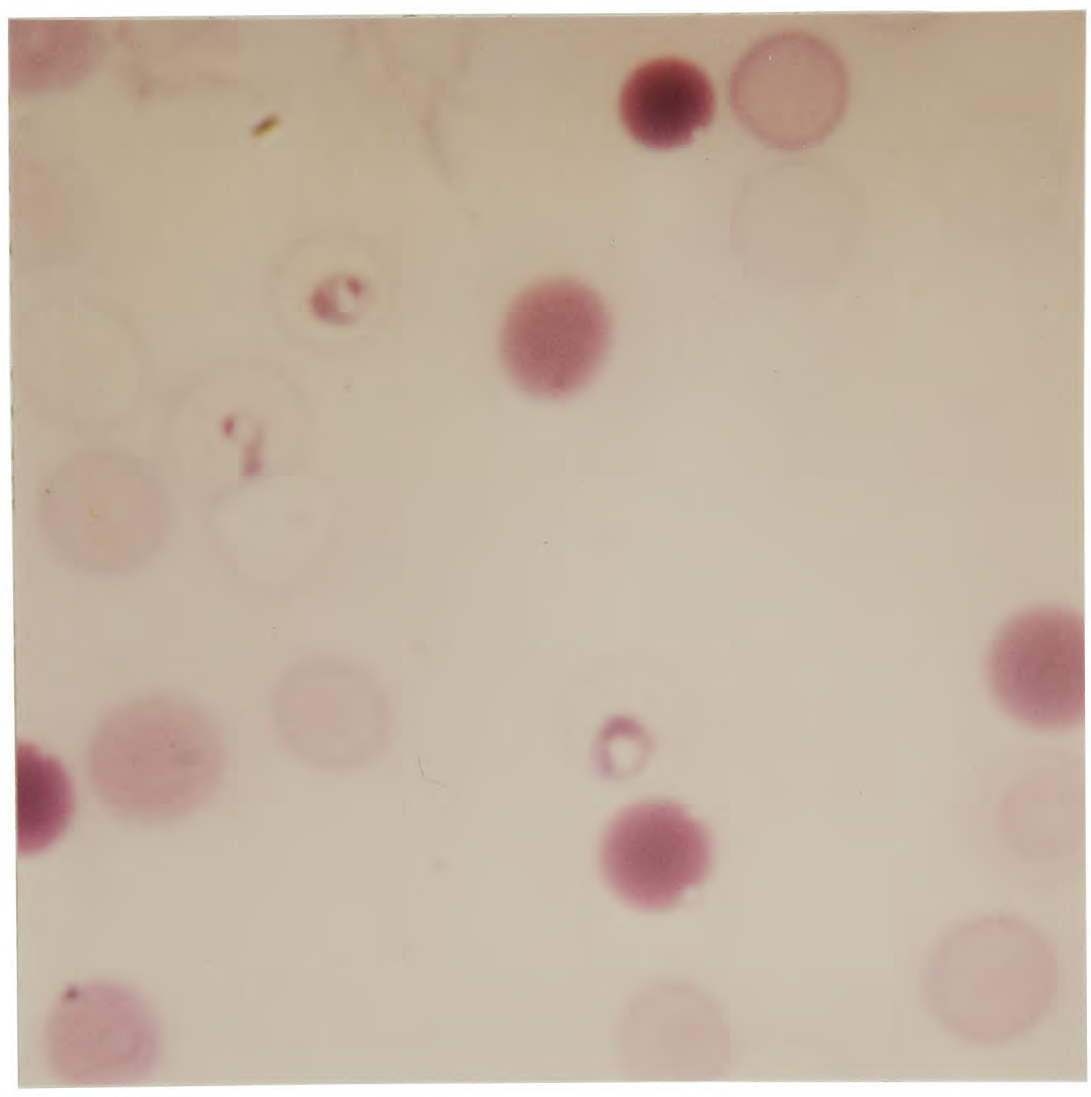


PLATE XIII Distribution of P. falciparum in cells containing Hb F which are OLDER than those containing Hb A (infant) (x 1700).

they were preferentially invaded, where they were of the same age as Hb A-containing cells there was an equal invasion of the cell types, but where the Hb F-containing cells were on average older, there was a paucity of parasites in them. These findings indicate that Hb F has no direct effect on the rate of invasion of red cells by P. falciparum and confirmed the preferential invasion of young cells.

C SHAKING OF CULTURES AND INVASION

During the course of this study the possibility was raised that the preference of P. falciparum for young cells was merely an artefact of the stationary culture system. It could be argued that young uninfected cells and cells containing mature parasites are more buoyant, and that after sedimentation these would be concentrated in the upper layers of red cells (calculated at approximately 50 deep in a microtissue culture well). Thus their proximity alone might lead to preferential invasion of the younger cell population.

In order to establish beyond any doubt that this was not the case, experiments were carried out in which cells were maintained in suspension by shaking in an orbital incubator (50 r.p.m.) at the time of parasite invasion. This speed of rotation was just sufficient to keep the cells in suspension. Similar cultures kept stationary served as controls.

In the first group of experiments (Table 16a), normal uninfected cells were first separated on the basis of age by centrifugation, and then fractions from the top, middle and bottom from such a specimen as well as whole blood were

Expt. No.	Donor cells	Recipient cells	Cell types present F/A	Parasites					
				Stationary			Moving		
				%	Multiply* infected cells (%)	F/A	%	Multiply infected cells (%)	F/A
1	7734	A 7711 F 7719	0.8	10.5	37	1.8	5.1	9	1.5
2	7730	A 7738 F 7733	1.1	5.0	35	1.9	3.4	8	1.5
3	7734	A 7738 F 7733	1.0	16.4	37	1.7	7.6	8	1.8
4	7729	A 7711 F 7719	1.0	4.3	19	2.5	1.8	4	2.2
5	7746	A 7738 F 7733	0.8	7.2	24	2.1	3.3	7	2.3
6	7768	A 7770 F 7761	0.7	11.0	25	2.2	8.3	8	2.3

*Multiply-infected cells are those containing 2 or more parasites and are expressed as a percentage of the infected cells i.e. not all of the cells present.

Table 16. Comparison of invasion by P. falciparum of cord and adult cells in stationary or moving cultures.

infected with donor cells as before (i.e. 3-5% schizont-infected cells). In the second experiment (Table 16b), equal numbers of adult and cord cells were infected with an inoculum of donor cells. In each case, 5 ml of the donor and recipient cell suspension were cultured in 100 ml sterile bottles (Flow Laboratories) which were either kept stationary or moving.

The results of these experiments (Tables 16 a and b) are similar. Both show that although shaking reduces the rates of invasion by parasites in all cultures and the percentage of cells with more than one parasite, the preference for young cells is maintained (the χ^2 test was significant for all the stationary and moving cultures where invasion of young and old red cells was compared, $p < 0.01$). Thus, the preferential invasion by P. falciparum was not found to be an artefact of the stationary culture system.

D PREFERENTIAL REMOVAL OF INFECTED Hb F-CONTAINING CELLS

1) Differential osmotic fragility of Hb F-containing cells in falciparum malaria

Another explanation for the paucity of parasites in the Hb F-containing cells of young infected infants was the possibility that these cells could be both preferentially invaded, but then preferentially removed from the circulation. Furthermore, the parasites in such Hb F-containing cells might be removed by the spleen and the cells then returned to the circulation as spherocytes, a process which is known to occur in malaria (Conrad, 1969). Any such decrease in surface area to volume ratio of the red cell would increase its

	Stationary					Moving				
Donor cells	7790									
Recipient cells	7789									
Blood fraction	Whole blood	Top (T)	Middle	Bottom (B)	T/B	Whole blood	Top (T)	Middle	Bottom (B)	T/B
Reticulocytes (%)	0.6	5.6	0.3	0.1	-	0.6	5.6	0.3	0.1	-
Parasites (%)	5.6	13.3	7.5	4.7	2.8	6.5	5.9	4.2	1.7	3.5
Multiply infected cells (%)	29	30	27	8	-	4	15	4	4	-

Table 16: Comparison of invasion by P. falciparum in stationary or moving cultures containing red blood cells separated on the basis of age by centrifugation prior to infection in vitro

osmotic fragility. Thus an experiment was undertaken to see if such a fragile population of cells existed and if so, whether this population contained a disproportionate amount of Hb F.

A young infant under the age of six months was admitted to the M.R.C. ward at Fajara, The Gambia. On alternate days blood specimens were taken to determine the osmotic fragility (Dacie and Lewis, 1975). Curves showing the osmotic fragility of such samples are shown in Figure 14. Compared to uninfected infant control blood there was, if anything, a marginal decrease in the osmotic fragility of the blood taken from the infant, all median corpuscular fragilities (i.e. % NaCl required to cause 50% lysis) being less than that of the control. There was certainly no indication at any time during the course of the illness of a population of particularly fragile cells.

In those test tubes from the osmotic fragility test where lesser degrees of lysis had occurred, the non-lysed cells were recovered by centrifugation, washed in PBS, and the percentage of Hb F was determined in each using the method of Singer et al (1951). There was no significant difference in the percentage Hb F found in whole blood and in any of the samples in which some osmotic lysis had occurred by using the Student's t distribution ($p > 0.10$) (Table 17). Therefore it appeared that, under these conditions, Hb F-containing cells were not more susceptible to osmotic lysis.

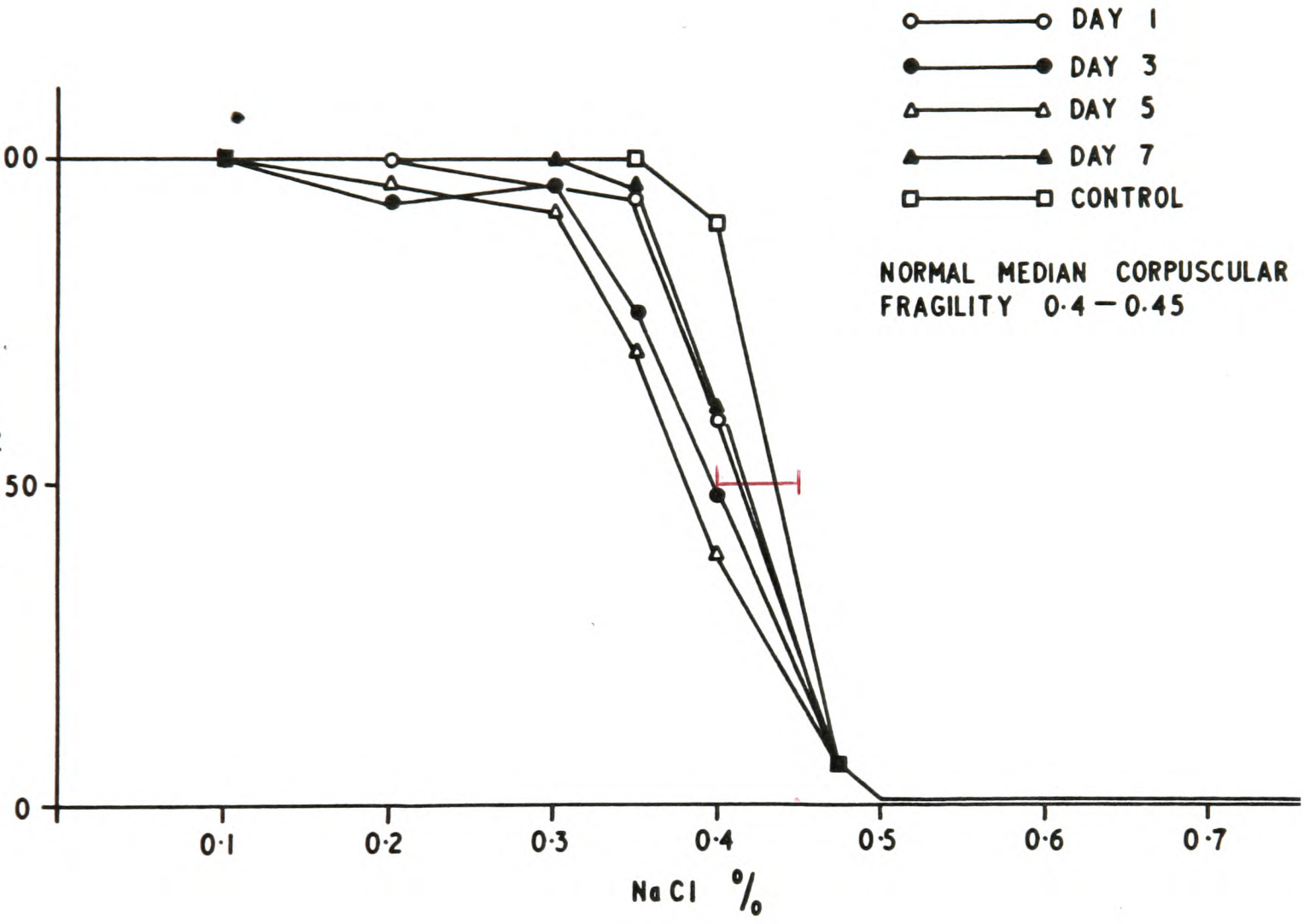


Figure 14 Osmotic fragility curves in an infant during the course of P. falciparum malaria.

Days after admission	Hb (gm/dl)	Parasites (%)	Reticulocytes (%)	Bilirubin (um/l)	Fetal haemoglobin (%) (% lysis in parenthesis)			
					Whole blood	Non lysed cells		
0	7.7	3.8	5.7	27.4	24.0 (0)	23.3 (7)	21.5 (24)	22.4 (61)
2	7.7	1.0	10.7	30.8	21.5 (0)	20.9 (6)	22.3 (16)	23.7 (43)
4	7.9	2.2	20.7	41.8	19.6 (0)	20.0 (7)	17.8 (43)	-
6	7.5	1.1	13.1	85.5	21.0 (0)	18.0 (4)	20.0 (24)	21.0 (61)
				Mean	21.5	21.0		
				± SD	±1.8	±1.9		

Table 17 Percentage Hb F in the remaining non-lysed cells after varying in vitro hypotonic lysis of blood taken from an infected individual.

2) Fetal haemoglobin levels during a malarial infection

Three young infants under the age of six months with mild falciparum malaria (< 50 parasites per high power field on thick smear) were admitted to the M.R.C. ward at Fajara. Blood specimens were taken daily by venepuncture or fingerprick. Haemoglobin levels, parasite counts, reticulocyte counts and bilirubin estimation were made by standard haematological methods as were fetal haemoglobin and haptoglobin levels. In all three cases, blood cultures and examination of stools for ova, cysts and amoebae were negative. The infants were treated with chloroquine (10 mg/Kg) by mouth before being discharged. Follow-up blood tests were carried out later in the outpatient department.

Table 18 and Figure 15 show that in the three cases, despite an initial fall in total haemoglobin levels, the gradual decline of Hb F levels was not disproportionate. Proof of active haemolysis is reflected in the raised bilirubin levels and the absence of haptoglobin, which returned after treatment in two cases (I and II). Active erythropoiesis is shown by the raised reticulocyte count. Absolute Hb F levels however showed only a gradual decline in two cases and were little changed in the third.

Thus, the overall results in this section on invasion of parasites into red cells showed that in young infants whose red cells contained an uneven distribution of Hb A and Hb F, there were more parasites present in the cells containing Hb A. Subsequent experiments indicated that there was a predilection shown by the parasite for metabolically

Patient No.	Days after admission	Total Hb (g/dl)	Hb F (g/dl)	Bilirubin (um/l)	Hapto-globin (mg/dl)	Reticulo-cytes (%)	Parasites (%)
1	0	6.9	3.1	95.8	0	2.6	0.7
	1	6.9	3.1	100.9	0	8.0	0.5
	2	7.0	2.9	80.4	0	11.0	0
	3	6.8	3.0	49.6	0	14.8	0.6
	4	6.9	2.4	35.9	0	11.2	0.4
	5	6.7	2.6	29.1	0	15.2	0.7
	6	6.2	2.2	46.2	0	14.2	1.0
→	7	5.3	1.7	39.3	0	14.5	1.0
	14	8.8	2.1	20.5	50	7.3	0
2	0	10.1	2.2	140.2	0	3.0	1.7
	1	9.8	2.2	88.9	0	4.6	0.4
	2	9.0	1.8	15.3	0	5.5	2.0
	3	9.2	2.0	123.1	0	4.8	0.3
	4	8.3	1.7	95.8	0	7.4	0.7
	5	8.9	1.7	42.8	0	5.8	0.2
	6	8.5	1.7	30.8	0	6.4	0.2
	7	9.2	2.1	-	0	10.0	0
→	8	9.1	1.4	51.3	0	5.0	0.5
	16	10.8	1.3	61.6	0	4.1	0
3	0	7.7	1.2	27.4	0	5.7	3.8
	1	6.9	-	-	-	10.2	2.4
	2	7.7	1.3	30.8	0	10.4	1.0
	3	8.4	-	-	-	11.3	1.0
	4	7.9	1.4	41.0	0	20.7	2.2
	5	7.2	-	-	-	13.6	1.7
→	6	7.5	0.8	85.5	0	13.1	1.1
	9	6.3	1.1	25.7	110	5.3	0
	35	10.6	1.2	-	-	2.0	0

→ Indicates when patient was treated (Chloroquine 5mg/Kg by mouth)

Table 18 Selected blood parameters in young infants with malaria caused by P. falciparum

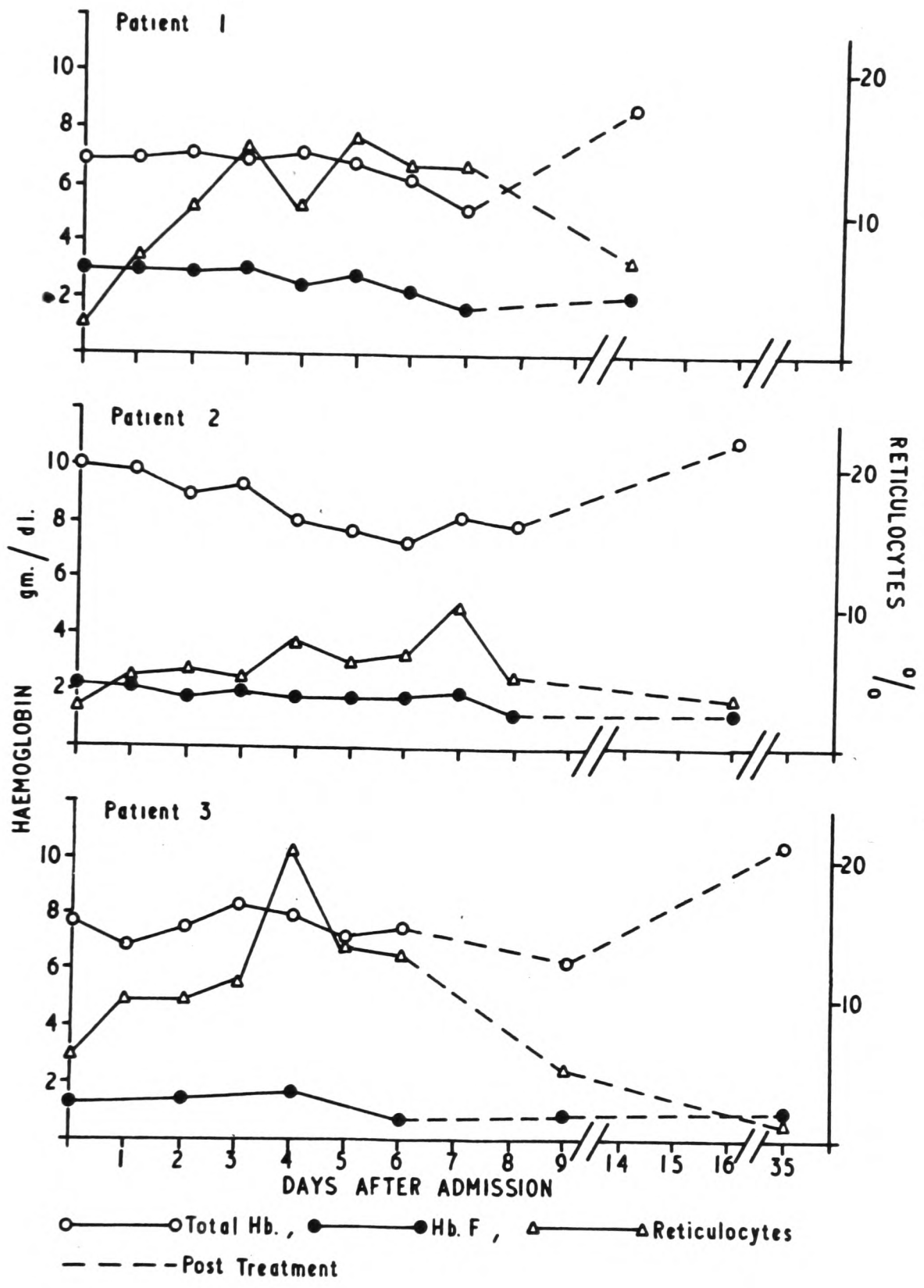


Figure 15 Total haemoglobin, Hb F and reticulocyte counts during the course of P. falciparum malaria in three infants.

active young red cells. Hb F was found to have no effect on the invasion of red cells by P. falciparum. Furthermore, no evidence of premature haemolysis of Hb F-containing cells in infected infants was found. In conclusion, it was felt that preferential invasion by P. falciparum of young red cells was sufficient to account for the initial interesting observation of a paucity of parasites found in the Hb F-containing cells of young infants.

CHAPTER 5

PARASITE DEVELOPMENT

From the results in the previous section, it appeared that the uneven distribution of parasites between Hb A and Hb F-containing cells of infected infants could be accounted for by the effect of cell age on parasite invasion without implicating Hb F. No correlation was found between Hb F and the invasive ability of the parasite. However, the process of parasite growth subsequent to successful invasion of cells containing Hb F had not been examined. Since the in vitro system allows easy assessment of development of parasites from the ring to schizont stage, such studies were undertaken to see if there was any difference between parasite growth in cells containing Hb F and those containing Hb A.

CELL AGE AND DEVELOPMENT

However, before examining growth in cells containing Hb F, it was important to establish if cell age on its own could affect parasite development since it had been found to alter parasite invasion so dramatically. Furthermore, in assessing growth of parasites in Hb F, cells of widely different metabolic age were to be included. Two of the experiments described in the previous chapter, where invasion of uninfected red cells separated into different age groups had been assessed (Expt. Nos. 3 and 4, Table 12), were used to examine parasite development. Following invasion, the parasites were maintained in culture and 36 hours later

growth was assessed both morphologically and by isoleucine incorporation (Table 19). It was found that parasite growth, as gauged microscopically or by radioisotope incorporation was similar in all cell fractions whether they were from the old, young, or whole blood cell fractions. Only minor differences are reflected in the difference between the mean growth score (MGS) of whole blood as compared to ıe various cell fractions (mean D = -0.1). There was no obvious trend between growth in cell fractions containing the young, average or old red cell populations and these results differed from the invasion process which was found to be related to the metabolic age of red cells. This clearly distinguished the invasion process from the subsequent development of the parasite once inside the red cell.

Hb F AND DEVELOPMENT

Experiments were carried out to compare the development of P. falciparum in Hb F-containing cells with those which contained Hb A. Using cells from naturally occurring conditions in which the Hb F is raised, parasite development was measured by a combination of methods including morphological assessment, incorporation of ^{3}H isoleucine and the weighing of paper cut-outs of photographic enlargements of individual parasites.

1. Cord cells

a) Mixed cultures of adult and cord cells

The four experiments in Table 20 show that in each of these mixed cultures of adult and cord cells, the more mature population of parasites was to be found in the fully eluted Hb A-containing cells. This is reflected in the difference in mean growth scores (D) between normal adult (A) and the

Experimental details	Expt. No	1				2			
	Donor cells	765				765			
	Recipient cells	7612				7613			
	Cell fraction	Whole blood	Top	Middle	Bottom	Whole blood	Top	Middle	Bottom
	Parasite (%)	5.4	8.3	5.0	1.9	5.2	6.2	4.7	3.3
Growth morphology	MGS	3.1	3.2	3.4	3.0	3.1	2.9	2.9	2.9
	D	-	-0.1	+0.3	-0.1	-	-0.2	-0.2	-0.2
Isoleucine incorporation	DPMx10^{-3}/well	8.2	12.4	11.0	3.1	9.4	10.4	7.0	6.0
	DPMx10^{-3}/% parasite	1.5	1.5	2.2	1.6	1.8	1.7	1.5	1.8
	R incorp	-	1.0	1.5	1.1	-	0.9	0.8	1.0

Control = whole blood
Test = particular cell fraction

Mean D = -0.1
Mean R_{incorp} = 1.1

Table 19 Growth of P. falciparum in red cells of different metabolic age.

Experimental details	Expt. No.	1		2		3		4	
	Donor cells	742		743		75111		7628	
	Recipient cells	742	741	743	741	75111	75117	7631	7629
	Cell type	A	F	A	F	A	F	A	F
Growth morphology	MGS	3.8	3.4	3.9	3.4	2.9	2.2	3.6	1.7
	D	-	-0.4	-	-0.5	-	-0.7	-	-1.9

Mean D = -0.9

Table 20 Growth of P. falciparum in cord and adult cells in the same cultures.

cord (F) cells - the mean value for these differences being -0.9 (a difference in the growth score of 1 would represent a difference of one stage of development). Plate XIV illustrates the presence of a smaller more immature parasite in a Hb F-containing cell compared to a normal adult cell. In addition to the retardation of parasite growth, more abnormal forms were seen in the cord cells, especially in experiments 3 and 4 (Appendix C). The results in experiment 3, where many of the parasites were still immature and hence the MGS even in the control cells is under 3, show that this retardation was evident even at the early stages of development and did not affect only mature parasites.

b) Cord and adult cells grown in parallel cultures

In other experiments (Table 21), the growth of parasites in cord and normal adult blood was compared, but in these experiments the control and test cells were maintained in separate culture wells (i.e. grown in parallel). Consequently, morphological assessment of growth did not require visual distinction between Hb A- and Hb F-containing cells. In all seven experiments carried out using this parallel culture method, there was clear retardation of parasite growth in the Hb F-containing cord cells (mean D = -0.6) (Table 21).

c) Growth in adult and cord cells assessed by ^{3}H isoleucine incorporation

Parasite development was assessed in red cells from three normal adults and five newborn Gambian infants using the same donor cells (Table 22). At 33 and 53 hours after

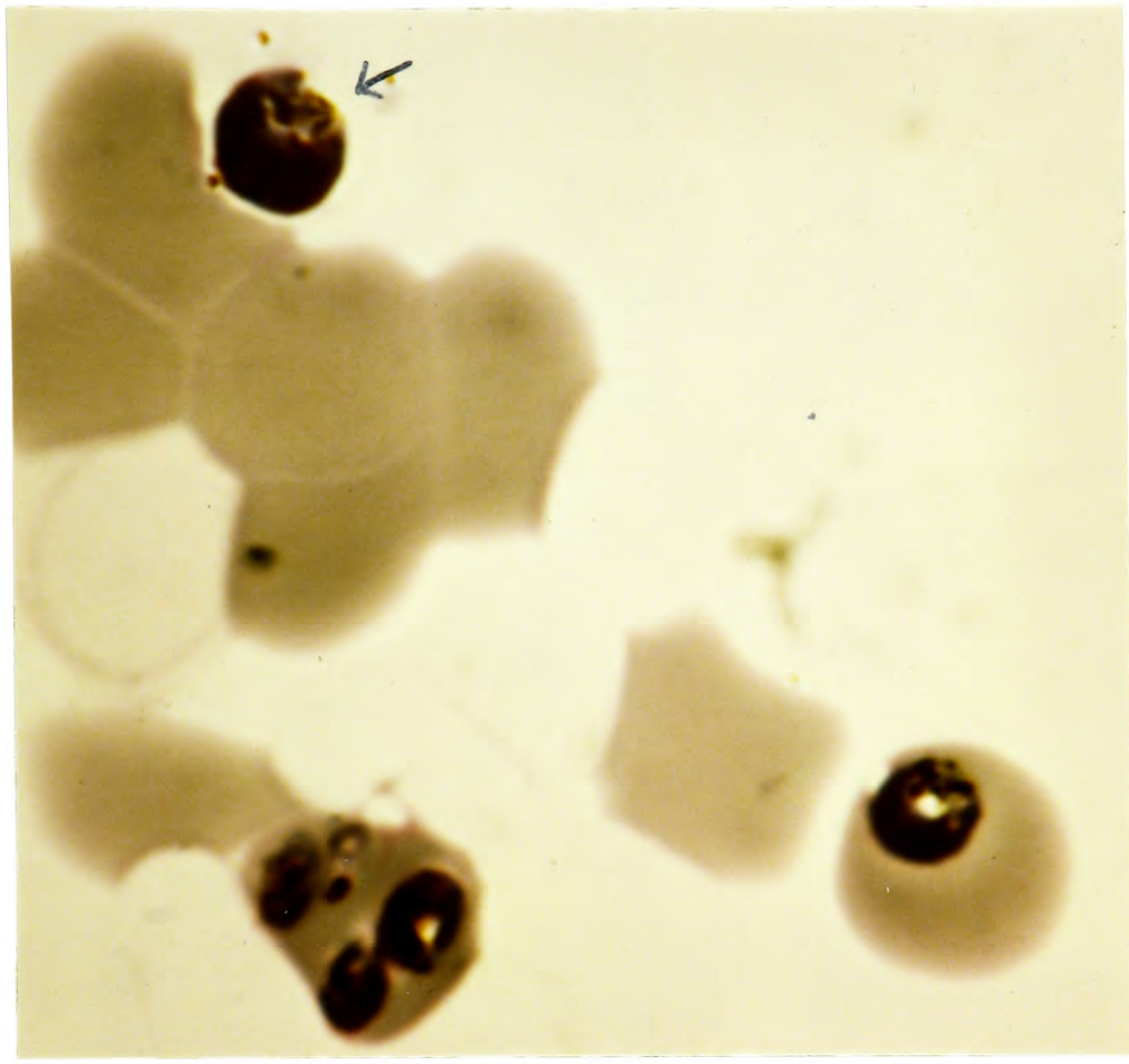


PLATE XIV Growth of P. falciparum in cord and adult cells showing a smaller parasite in a Hb F-containing cell than in one containing Hb A (x 2500). (arrowed)

Experimental details	Expt. No	1		2		3		4		5		6		7	
	Donor cells	764		76113		7661		76112		76115		7662		7686	
	Recipient cells	7610	7611	76100	76105	7653	7651	76100	76105	76100	76105	7660	7659	7683	7685
	Cell type	A	F	A	F	A	F	A	F	A	F	A	F	A	F
Growth morphology	MGS	3.4	3.1	3.2	2.7	3.7	3.1	3.4	2.7	3.2	2.5	3.2	2.4	3.0	2.1
	D	-	-0.3	-	-0.5	-	-0.6	-	-0.7	-	-0.7	-	-0.8	-	-0.9

Mean D = -0.6

Table 21 Growth of P. falciparum in cord and adult cells in parallel cultures.

subculture, wells containing infected cells were labelled with 1 μCi ^{3}H isoleucine in triplicate and uninfected cells were labelled in duplicate. Incorporation by parasites was measured over the subsequent 12 h period. Parasite counts at the onset of these pulse periods made it possible to relate the incorporation of isoleucine to the percentage viable (i.e. excluding abnormal forms) parasites present.

The results (Table 22) show that the uptake of isotope by the parasites in the wells with Hb A-containing cells during both labelling periods is higher than that of the parasites in any of the cord bloods. This difference is even more striking when related to the parasite count where it can be clearly seen that incorporation per parasite in adult cells is in most cases over twice that in cord cells (mean R incorp. = 0.4).

Morphological assessment of growth at 53 h (Table 22) confirmed this retardation of development in cord red cells (mean D = -1.1). In addition, more abnormal forms were found to be present in these cells.

d) Growth in adult and cord cells assessed by weighed cut-outs of parasite photographs

Donor cells were added to a mixture of equal numbers of normal adult and cord cells. The mixture was then incubated for 45 h after which growth of parasites in the adult and cord cells was assessed by weighing cut out enlarged images of the parasites in each cell type. A bar histogram of the relative weights of 114 parasites in each group (A and F) is shown in Figure 16. It can be seen that the heavier and therefore larger parasites were to be found in the Hb A-

Table 22 Incorporation of ^{3}H isoleucine by *P. falciparum* in cord and adult cells.

Experimental details	Donor cells	7639							
	Recipient cells	7647	7645	7646	7640	7642	7643	7644	7641
	Cell type	A	A	A	F	F	F	F	F
Growth morphology (53 h)	MGS	3.9	3.5	3.3	2.8	2.6	2.6	2.5	2.1
		(mean) 3.6							
	D	+0.3	-0.1	-0.3	-0.8	-1.0	-1.0	-1.1	-1.5
Isoleucine incorporation a) 1st pulse period (33-45 h)	dpmx10^{-3} per well	50.8	41.7	35.0	34.1	23.7	25.8	32.9	27.8
	Viable parasites %	8.6	9.3	9.1	16.2	12.7	14.9	19.4	15.3
	dpmx10^{-3} per % parasites	5.9	4.5	3.8	2.1	1.9	1.7	1.7	1.8
		(mean) 4.7							
	R incorp	1.3	1.0	0.7	0.4	0.4	0.4	0.4	0.4
b) 2nd pulse period (53-65 h)	dpmx10^{-3} per well	44.8	49.9	45.2	40.7	40.3	43.7	29.5	37.2
	Viable parasites %	5.4	4.6	4.2	10.2	10.4	10.0	6.7	9.5
	dpmx10^{-3} per % parasites	8.3	10.8	10.8	3.9	3.9	4.4	4.4	3.9
		(mean) 10.0							
	R incorp	0.8	1.1	1.1	0.4	0.4	0.4	0.4	0.4

F cells Mean D = -1.1 Mean R incorp (a) = 0.4
(b) = 0.4

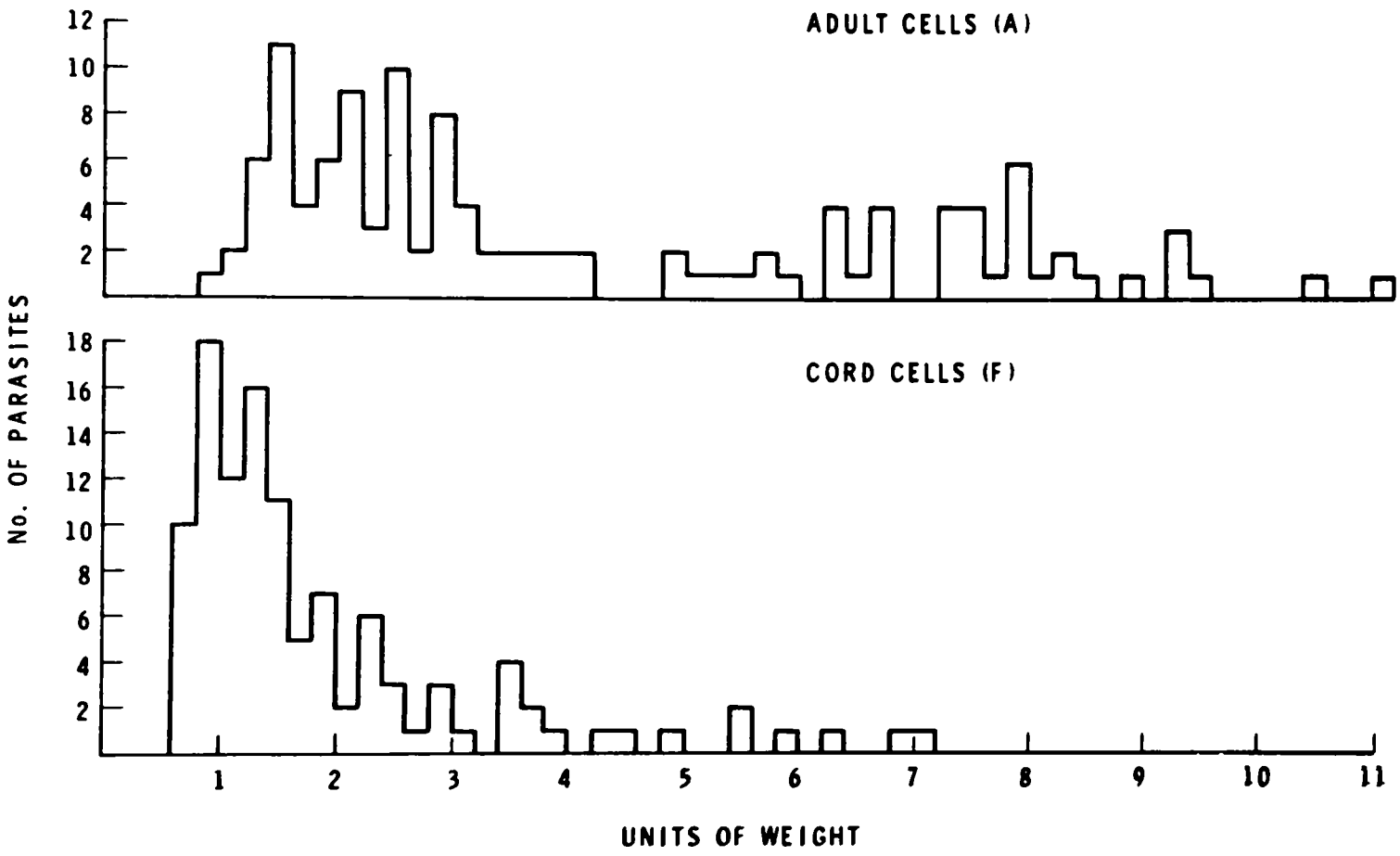


Figure 16 Size distribution of P. falciparum in a mixture of cord and adult cells (114 parasites in each group).

containing cells.

2) Compound $\beta^{o}/\delta\beta^{o}$ thalassaemia cells

In two experiments, growth in such cells which contain 100% Hb F showed a marked retardation of development when compared to normal adult cells (Table 23). The relatively large differences in growth scores between the controls and those cells under test (-1.0 and -1.3) are particularly relevant, as the average maturity of the parasites in the control cells is low (i.e. a MGS of 3.0 in both of the controls corresponds to the majority of parasites being in the small trophozoite stage). When assessed at a later stage in the culture, most of the parasites in the $\beta^{o}/\delta\beta^{o}$ thalassaemia cells were abnormal, whereas the parasites in the controls continued to grow.

3) British form of hereditary persistence of fetal haemoglobin (HPFH)

The growth of parasites in cells from the British form of HPFH is shown in Table 24. In all six cases there was clear retardation of growth in the cells containing most stainable Hb F. The mean difference in growth scores between the A and F cells was -0.9, and was of a similar order to the retardation seen in cord cells. The value for the intermediately staining cells lay between that of the A and F cells.

The relative retardation of parasite growth in the Hb F-containing cells from these British HPFH cases was further confirmed by using the method of weighing cut-outs of photographed parasites (Figure17) which shows the size distribution of 143 parasites in each of the A and F groups.

Experimental details	Expt. No	1		2	
	Donor cells	75111		75110	
	Recipient cells	75111+	7527	75110+	7527
	Cell type	A	F	A	F
Growth morphology	MGS	3.0	2.0	3.0	1.7
	D	-	-1.0	-	-1.3

Mean D = -1.2

\+ Donor and recipient cells cultured directly into microtissue culture plates. Controls (Hb A-containing cells) provided by uninfected donor cells.

Table 23 Growth of P. falciparum in cells from a $\beta^{o}/\delta\beta^{o}$ thalassaemia compound heterozygote.

Experimental details	Expt. No	1			2			3			4			5			6		
	Donor cells	7649			76113			76115			7613			7646			7545		
	Recipient cells	7544			76104			76104			762			7544			7544		
	Cell type	A	I	F	A	I	F	A	I	F	A	I	F	A	I	F	A	I	F
Growth morphology	MGS	3.5	3.2	3.0	3.5	2.9	2.7	2.9	2.5	2.1	3.7	3.0	2.7	3.1	2.6	2.0	3.9	2.5	2.5
	D	/	-0.3	-0.5	/	-0.6	-0.8	/	-0.6	-0.8	/	-0.7	-1.0	/	-0.5	-1.1	/	-1.4	-1.4

Mean D (I) = -0.7

(F) = -0.9

Table 24 Growth of P. falciparum in cells of the British form of hereditary persistence of fetal haemoglobin (HPFH)

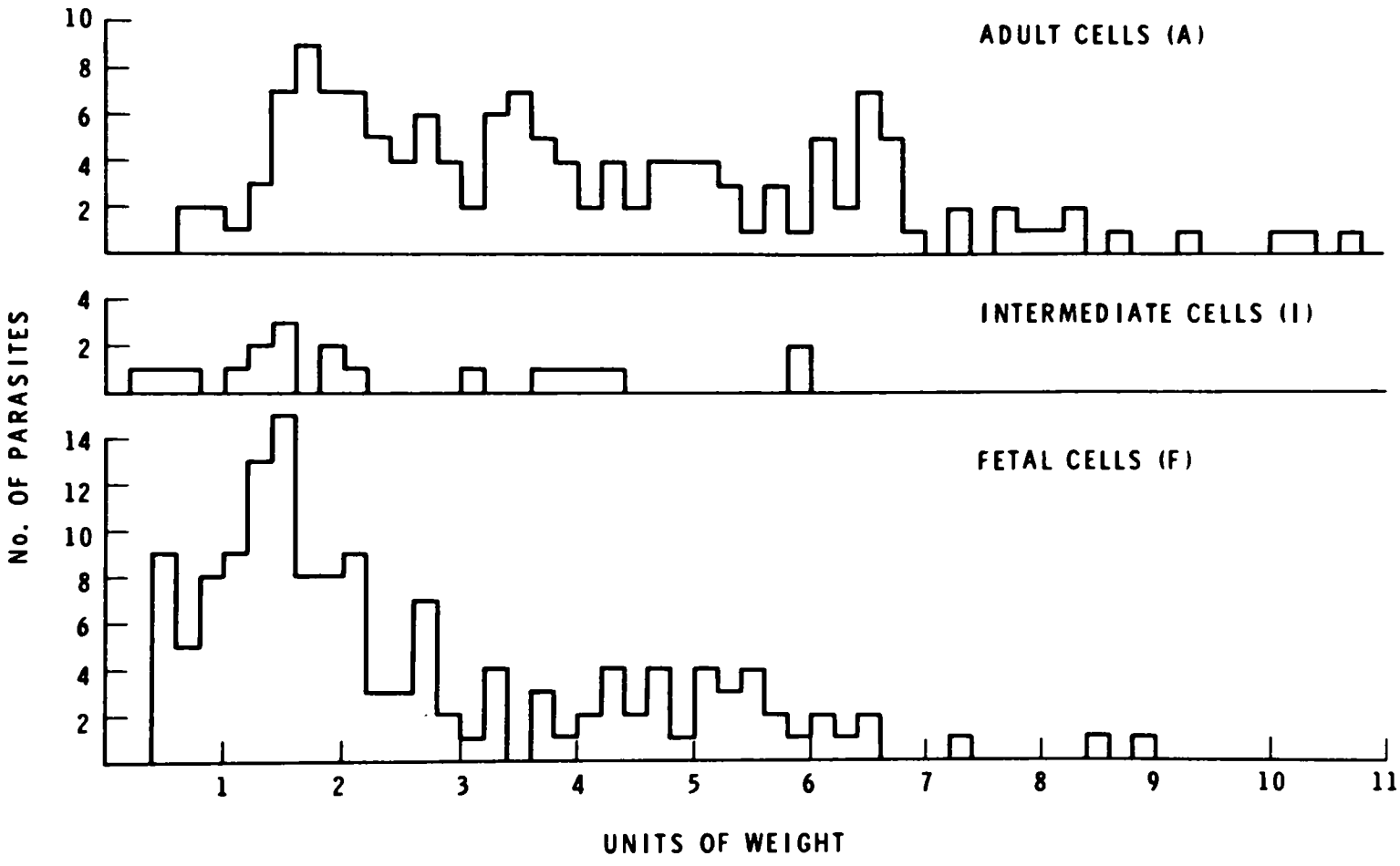


Figure 17 Size distribution of P. falciparum in the cells of the British form of hereditary persistence of fetal haemoglobin (HPFH) (143 parasites in each of the A and F cell groups).

It can be seen on the figure that there is a cluster of parasites of small size in the cells containing Hb F, whereas in the eluted Hb A-containing cells numerous large parasites are present.

4) African forms of hereditary persistence of fetal haemoglobin (HPFH)

The growth of parasites in these cells compared to those in a parallel culture of normal adult cells is shown in Table 25. Three different infected bloods were tested with the same specimen of HPFH blood. In each experiment, a retardation of growth was seen in the Hb F-containing cells, though not as marked as in cord blood as judged by the difference between control and test mean growth scores which averages about -0.6 (this figure was closer to -1.0 in experiments involving cord cells).

5) Infant blood

Blood obtained from infants infected with P. falciparum was washed twice with PBS to remove homologous plasma and cultured directly into microtissue culture plates.

The growth of parasites assessed morphologically in cells from young infants with varying amounts of Hb F is shown in Table 26. In experiments 2 and 3, assessment of growth was made twice during the culture of parasites from the same blood. The results show that in all cases there is retardation of growth in the Hb F-containing cells (mean D = -1.0). In the intermediate (I) staining cells the degree of growth retardation lies between those of the A and F group (mean D = -0.6). This retardation is shown

Experimental details	Expt. No.	1		2		3	
	Donor cells	76113		76112		76115	
	Recipient cells	76100	76109	7660	76109	76100	76109
	Cell type	A	F	A	F	A	F
Growth morphology	MGS	3.7	3.2	3.2	2.6	3.4	2.8
	D	/	-0.7	/	-0.6	/	-0.6

Mean D = -0.6

Table 25 Growth of P. falciparum in cells of the African form of hereditary persistence of fetal haemoglobin (HPFH)

Experimental details	Expt. No	1			2						3					
	Infected cells	75219			75224						75211					
	Time in culture	After reinvasion			Before invasion (20 h)			(43 h)			Before reinvasion			After reinvasion		
	Cell type	A	I	F	A	I	F	A	I	F	A	I	F	A	I	F
Growth morphology	MGS	2.6	2.1	2.0	2.8	3.1	2.7	5.0	3.7	3.5	2.6	1.9	1.9	2.8	2.0	1.8
	D	/	-0.5	-0.6	/	+0.3	-0.1	/	-1.3	-1.5	/	-0.7	-0.7	/	-0.8	-1.0

Mean D (I) = -0.6

(F) = -1.0

Table 26 Growth of P. falciparum in cells of young infants.

in experiment 2 to occur at both earlier and later stages of the growth cycle, but this difference increases with maturation of parasites. A further advantage of these experiments is that they could be subcultured directly in microtissue culture trays after obtaining the infected blood from infants, because some parasites were already present in cells containing Hb F. The parasites in these experiments had therefore spent comparatively shorter times in the *in vitro* culture system. In addition, direct culture in these cells facilitated the assessment of growth both before and after invasion had taken place *in vitro*. Experiment 3 shows that the observation of growth retardation in cells containing Hb F holds for both generations of parasites, i.e. those that had invaded red cells *in vivo* and those where invasion had occurred *in vitro*. The difference in growth scores (D) between the A and F cells was moreover of a similar order both before and after invasion *in vitro*.

GAMETOCYTE DEVELOPMENT

During the course of the experiments with the asexual forms of *P. falciparum*, the appearance and development of sexual forms (gametocytes) was noticed in many of the cultures. Their appearance often heralded the demise of the asexual forms present, whilst the gametocytes persisted, sometimes for many days in culture. Data on these gametocytes is included because in some cultures Hb F-containing cells were found to contain them. This indicated that they must have arisen from merozoites released *in vitro* by the erythrocytic forms of the parasite.

The gametocyte develops quite differently from the

asexual forms (Hawking et al, 1968; Smalley and Sinden, 1977). By a still unknown stimulus, they grow into a crescentic female (macro) gametocyte with a compact nucleus and pigment granules, and a smaller male (micro) gametocyte with a less compact nucleus and more diffusely distributed pigment. Unlike the asexual forms, the early stages of differentiation of gametocytes of P. falciparum from merozoites do not usually occur in the peripheral blood but in the deep tissues. Morphologically mature or near mature gametocytes are released into the peripheral circulation only after nine or 10 days development. However, they do not seem to be involved in the clinical manifestations of the disease (Garnham, 1966) and hence cannot be implicated in the malaria hypothesis. However, since they are essential for the transmission of the disease via the mosquito, they are of paramount epidemiological significance.

The appearance of gametocytes in cells containing Hb F Schizont-infected cells were subcultured with a mixture of normal adult cells from the umbilical cord of a healthy newborn Gambian (Table 27). Careful examination of the donor cells showed that no apparent sexual forms were present in the initial infected sample. Invasion of the uninfected recipient cells commenced within a few hours of the culture being initiated. There was more than a threefold increase in parasite numbers at 17 h. By 77 h, a third generation of parasites had arisen with a decrease in the density of asexual parasites. It was at this stage that immature gametocytes were first observed, both in Hb A and Hb F-containing cells. After 170 h in culture, 200 µl of medium was replaced as the supernatant was clearly acidic. At 170 and 216 h, gametocytes at different stages of development

Time (h)	Parasite morphology				Cells counted	Parasites (%)
	R	T	S	G		
0	0	0	18	0	513	3.4
	Schizogony and invasion					
17	128	0	0	0	1064	12.0
56	0	54	0	0	532	10.2
	Schizogony and invasion					
77	39	15	6	1	878	6.9
89	8	11	3	9	862	3.6
170	No viable asexual stages seen			16	2000	0.8
216	"			17	2000	0.9
296	"			16	2000	0.8

Table 27 Appearance of gametocytes of P. falciparum in cultures with both Hb A- and Hb F-containing cells.

were distinguished. Frequently two and very occasionally three gametocytes were present in a single cell (Plate XV). At this time no morphologically normal asexual forms were recognisable.

Hb F and gametocyte development Assessment of gametocyte growth was made during this period. It could be clearly seen that gametocytes in the Hb F-containing cord cells were frequently smaller than those in the Hb A-containing red cells. This was confirmed by measuring the weights of enlarged photographs of 31 gametocytes in each cell type. The gametocytes in the Hb F-containing cells were noticeably smaller (Fig.18, Plate XV). This provided further evidence that Hb F-containing cord cells provide a less favorable environment for the parasite, both in its asexual and sexual forms.

Brief summary of the findings

Thus, the overall results on development of P. falciparum in cells containing Hb F showed that in all cells containing this haemoglobin, regardless of their source or age, there was a retardation of parasite development. This effect was found on both the sexual and asexual forms of the parasite and correlated with both the levels of Hb F present and the time spent in culture. It was also established that, unlike invasion where the parasite preferred metabolically young cells, growth was unaffected by the age of the red cell in which the parasite was contained. This clearly distinguished two separate processes; parasite invasion into red cells and subsequent growth of parasites once inside red cells.

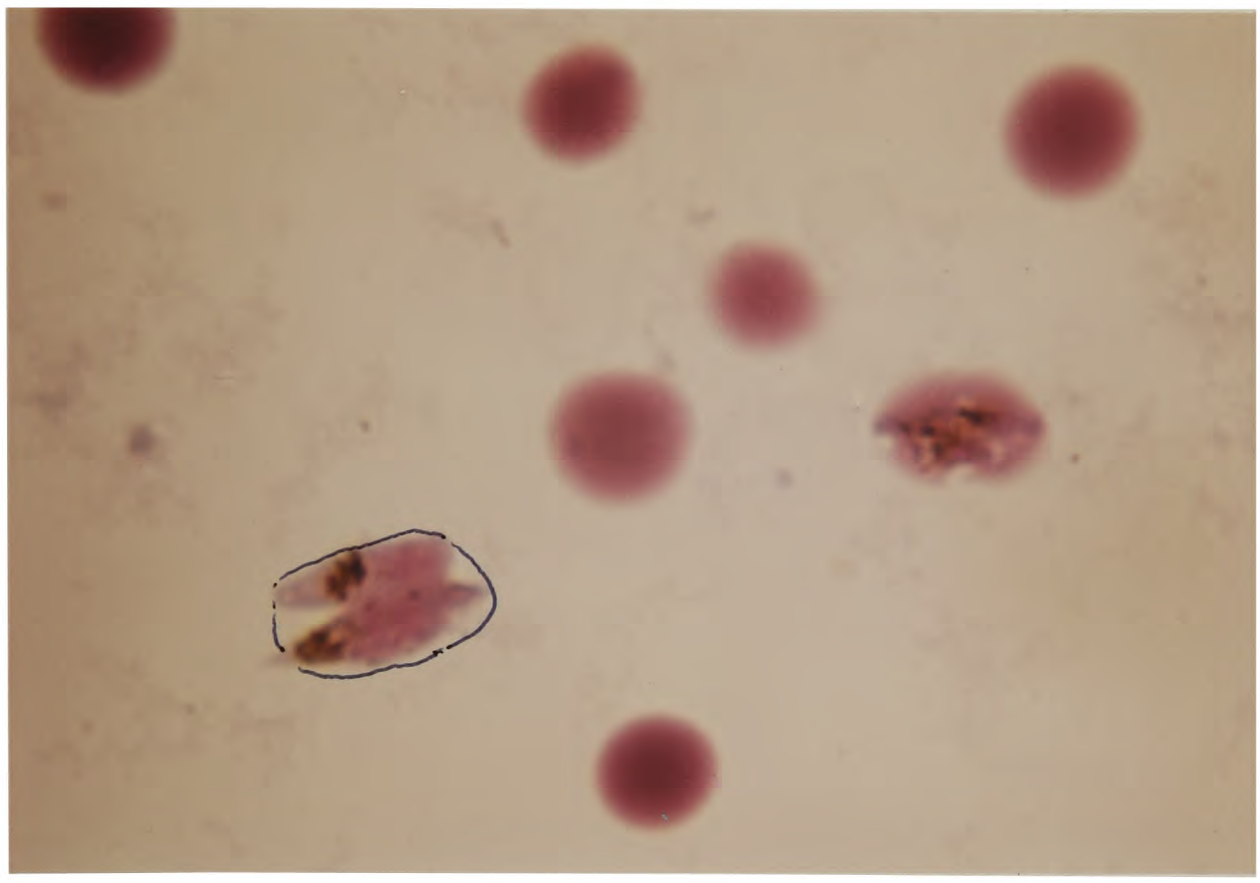


PLATE XV Gametocytes of P. falciparum in both Hb A and Hb F-containing cell. Note the gametocytes in the Hb F-containing cell are far smaller than those in the Hb A-containing cell (x 1500).

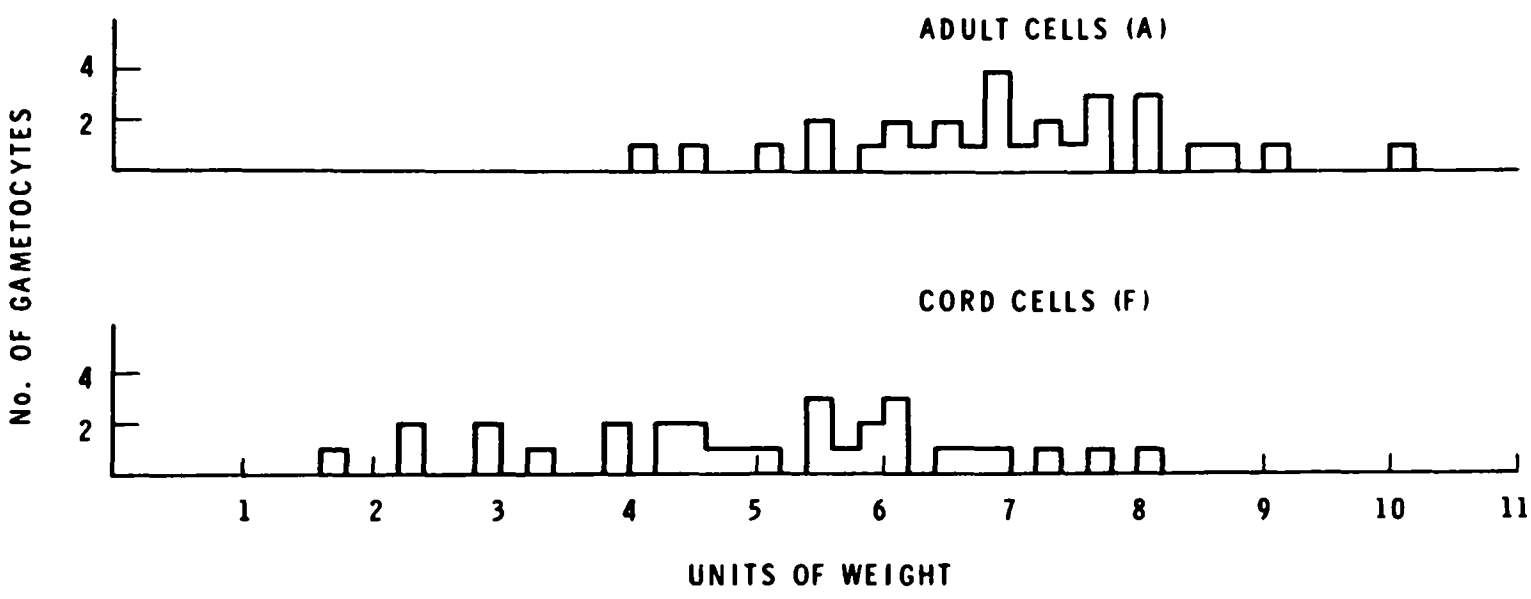


Figure 18 Size distribution of the gametocytes of P. falciparum in a mixture of cord and adult cells (31 parasites in each group).

CHAPTER 6

THE MECHANISM OF RETARDATION OF PARASITE GROWTH IN CELLS CONTAINING FETAL HAEMOGLOBIN

There seemed to be two likely explanations for the retardation of parasite growth seen in cells containing Hb F. Firstly, it was thought possible that the relatively high affinity Hb F has for oxygen might deprive the parasite of its own requirements. Secondly, the differences in structure of Hb A and Hb F might lead to differences in their degradation by malarial parasites.

Whatever the mechanism of growth retardation, it also seemed relevant to look at parasite multiplication after a period of growth in the Hb A- or Hb F-containing cells. This required observation of third generation rings in the limited short-term-culture system. However, it was important, since the effect observed had been that of growth retardation rather than inhibition, and the overall effect was unclear. One further problem tackled was an attempt to confirm in vitro whether the presence of transferred passive immunity in the serum of newborns living in areas endemic for malaria was sufficient to account for the observed resistance to the disease. Simple experiments were therefore carried out in this section in an attempt to answer some of these questions.

A OXYGEN AND PARASITE GROWTH

The effects of a lowered oxygen tension on parasite growth

Initially, since sophisticated equipment was not available, cultures were carried out under anaerobic conditions, similar to the methods employed for routine bacteriological studies. The culture trays were placed in plastic jars under conditions of anaerobiasis using the Complete GasPak system (Becton Dickinson, U.K.). In this system, 10 ml of water is poured into a commercially prepared sachet from which hydrogen and carbon dioxide are evolved. In the presence of a platinum catalyst, the oxygen combines with the hydrogen to form water to create anaerobic conditions. Carbon dioxide (5%) is also generated from a tablet of sodium bicarbonate and citric acid activated by the water. The culture conditions are judged anaerobic by a methylene blue indicator included inside the jar, which turns white in the absence of oxygen. At the commencement of a period of culture of 12 h, the blood cells were bright red but by the end they could be seen to be darker and more similar to venous blood than the bright red colour of the cells in a comparable aerobic culture. This indicated that the cells at the bottom of the culture wells were at least exposed to a lowered oxygen tension, at least towards the end of such a period.

Initially, infected blood was set up directly into microtissue tray wells, labelled with ^{3}H isoleucine in triplicate and cultured aerobically or under anaerobic

conditions for 12 h periods. At the end of this period, smears were taken from both aerobic and anaerobic cultures and the incorporation per well was counted. This was repeated up to 72 h. The cumulative ^{3}H isoleucine incorporation in three such experiments is shown graphically in Figure 19. Blood smears prepared at the same time were used to determine the duration of the process of invasion of uninfected cells.

In all cases, the incorporation of ^{3}H isoleucine was higher in cultures maintained in the anaerobic jars compared to those grown aerobically. These differences arose mainly during the growth phase of the parasite cycle rather than during parasite invasion, when it can be seen from the graphs that incorporation of cultures under the two conditions runs more or less parallel. The morphological data for these three experiments also confirm more favourable parasite growth when placed in anaerobic jars for periods of 12 h at a time (mean D = +0.9) (Table 28).

Since it was felt that true anaerobiasis might not be achieved within the layer of red cells at the bottom of the microtissue culture wells in the relatively short time of 12 h, the time period of undisturbed culture was increased to 48 h. When growth was assessed in five such experiments, it was apparent that the parasites grown in anaerobic jars for 48 h had clearly not developed. Parasites grown aerobically, however, had developed to schizonts, and given rise to a new generation of morphologically normal rings, both small and large (Plate XVIa). In the anaerobic culture jars, the parasites had accumulated large

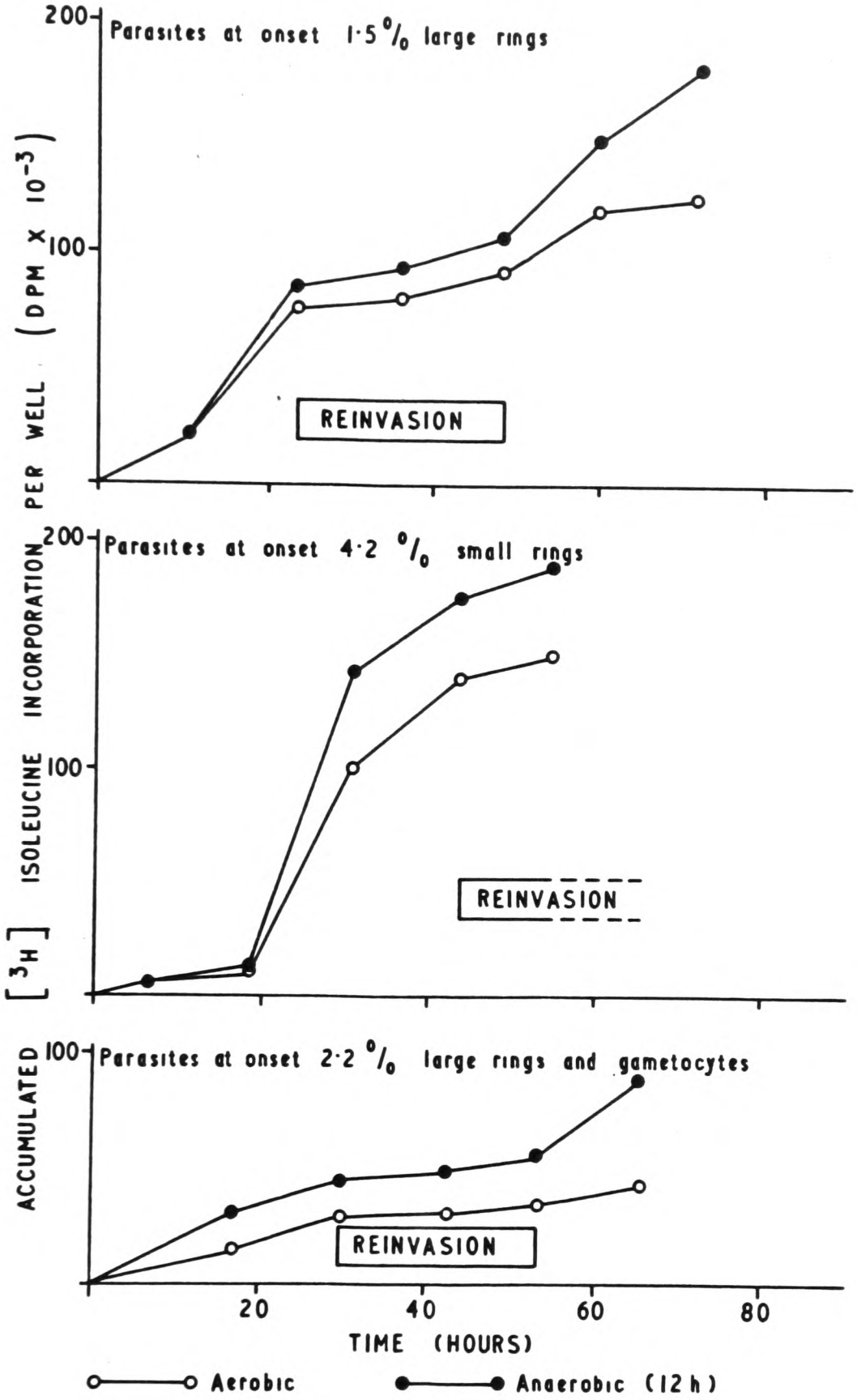


Figure 19 ^{3}H isoleucine incorporation by P. falciparum in either aerobic conditions or under low oxygen tensions.

Experimental details	Expt. No	1		2		3	
	Infected cells	7673		7675		7667	
	Time (h) after subculture	19 (Before reinvasion)		66 (After reinvasion)		72 (After reinvasion)	
	Conditions	Aer	Anaer	Aer	Anaer	Aer	Anaer
Growth morphology	MGS	3.4	3.9	2.2	3.2	2.8	4.0
	D	/	+0.5	/	+1.0	/	+1.2

Mean D = +0.9

Table 28 Comparison of growth of P. falciparum under aerobic conditions and low oxygen tensions

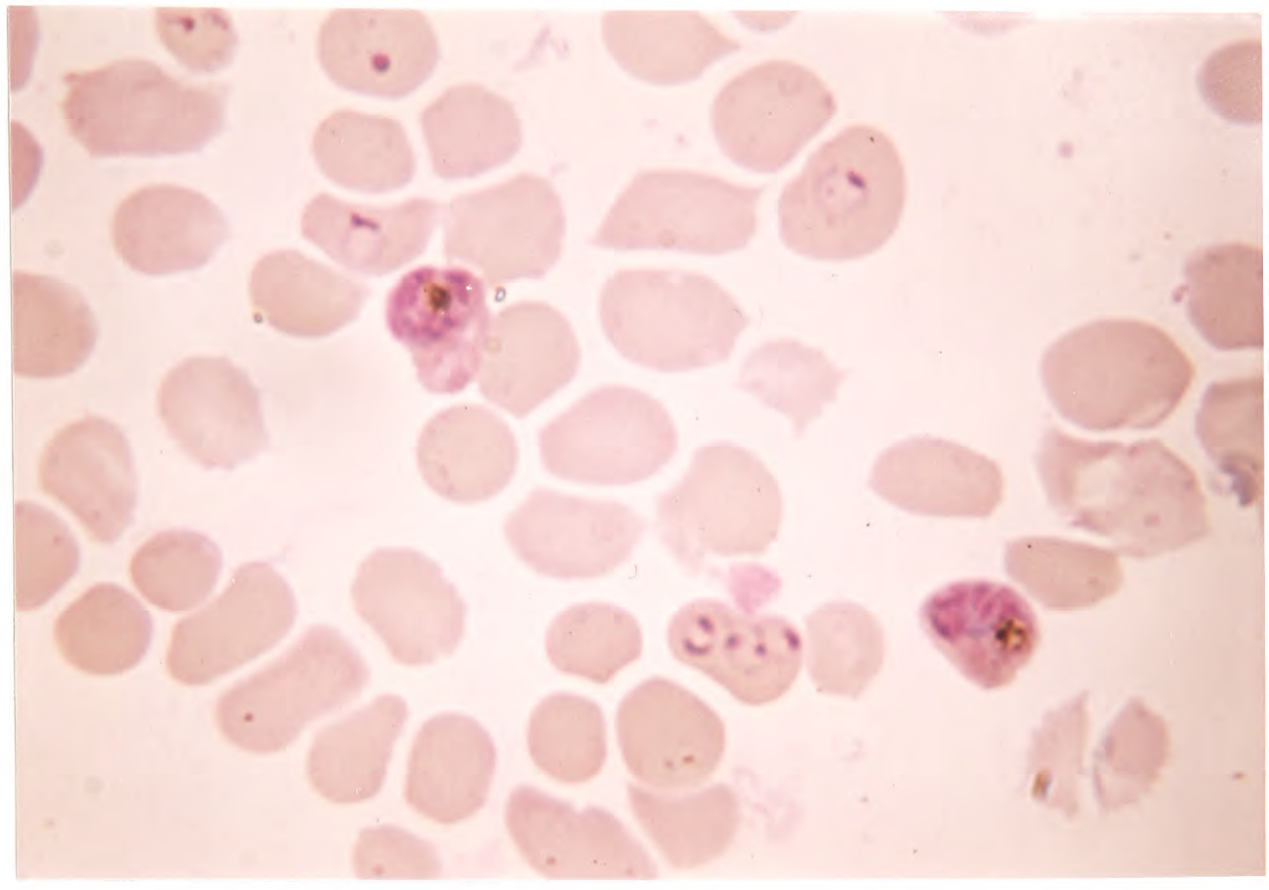


PLATE XVIa Growth of P. falciparum after 48 h of undisturbed culture under aerobic conditions (x 1500)

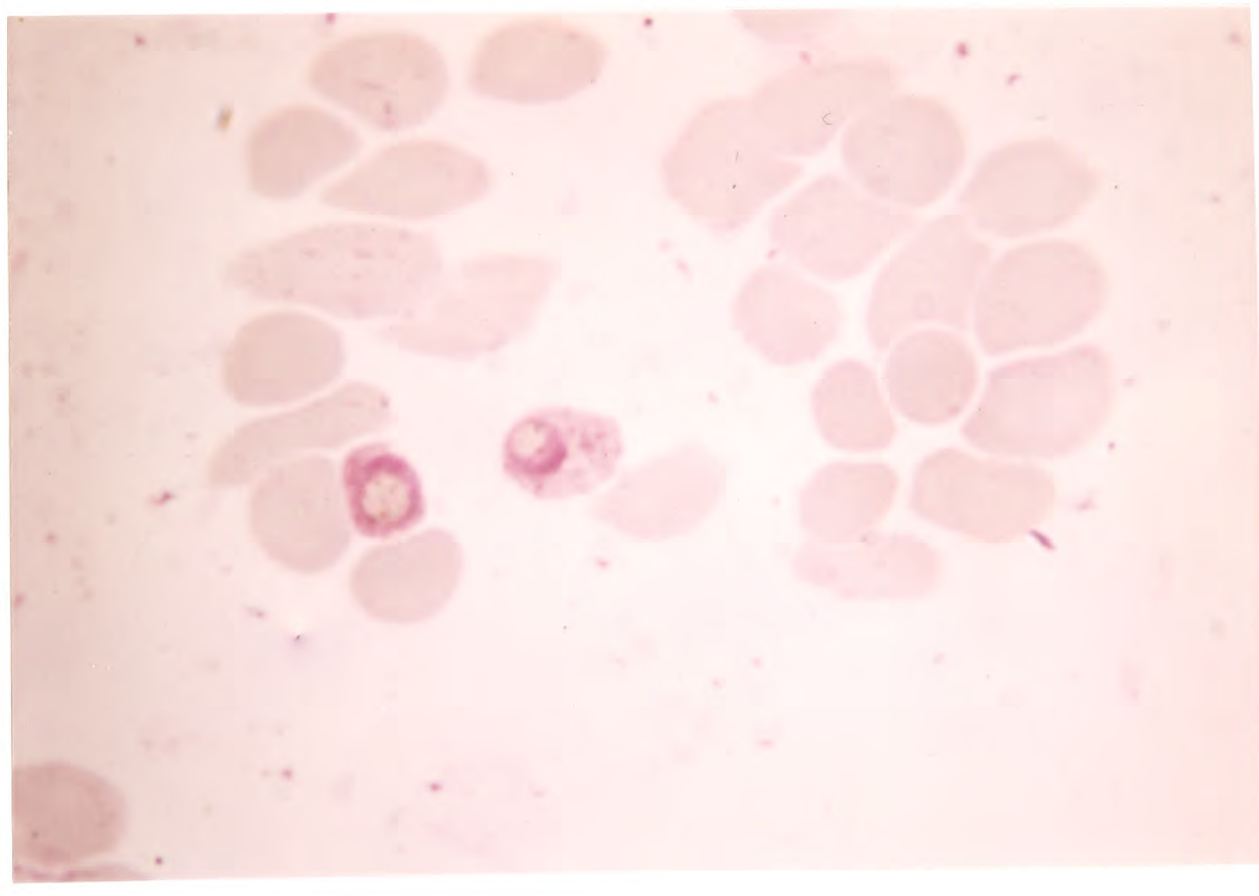


PLATE XVIb Growth of P. falciparum after 48 h of undisturbed culture under anaerobic conditions (x 1500).

amounts of pigment, appeared abnormal, and no normal schizonts were seen (Plate XVIb). Incorporation of ^{3}H isoleucine in such cultures was consistently greater in the cultures under aerobic conditions (Table 29). Using the 't' test on pairs, this was statistically significant ($0.05 > p > 0.02$). This was despite the fact that in these aerobic cultures invasion of red cells had occurred in which many merozoites labelled with isotope must have failed to invade new cells. Thus, in the prolonged absence of oxygen, parasites had failed to develop. However, a clear indication for oxygen as a requirement for parasite growth could not be confirmed as the inhibition of growth might, for example, have been due to the change in pH resulting from the accumulation of lactate under anaerobic conditions.

The oxygen tensions present in the experiments involving anaerobic jars

During the last visit to the Gambia the conditions of oxygen tension in these anaerobic jars were more clearly defined, using an oxygen monitor and electrode (Becton Dickinson, U.K.). The change in percentage oxygen was measured, both by suspending the electrode in the gas above the medium inside such a jar, and by placing it in the medium. The electrode was immersed to a depth of 8 mm in a 10 ml glass beaker containing 8 ml supplemented medium 199. This was the depth at which cells lie in the microtissue culture system (the electrode was too large to be placed in a well of a culture plate). Figure 20 shows the change in oxygen tension with time in three experiments, both above the culture medium and in it. The results confirmed the suspicion

Expt. No.	Parasites (%)	dpm x 10^{-3} per well		
		Aerobic*	Anaerobic	R incorp
1	1.4	21.0	16.1	0.8
2	1.8	16.3	12.0	0.7
3	1.9	11.9	6.2	0.5
4	2.5	22.9	16.7	0.7
5	4.2	53.1	38.2	0.7
6	5.1	40.1	12.7	0.3

* Smears taken from aerobic cultures showed viable mature parasites whereas smears from anaerobic cultures showed that all parasites were abnormal at 48 h.

Table 29 Incorporation of ^{3}H isoleucine by P. falciparum in 48 h undisturbed aerobic or anaerobic cultures.

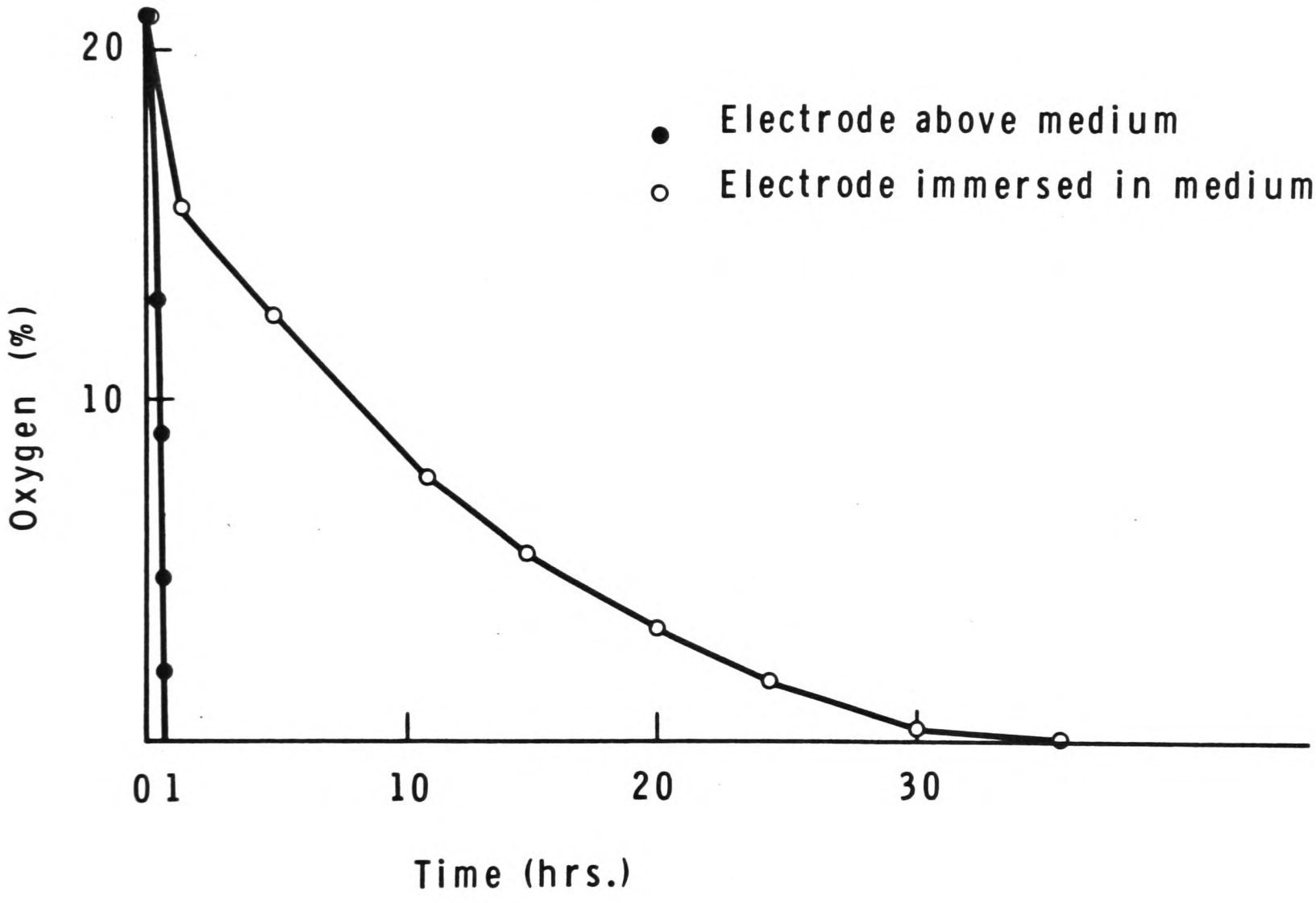


Figure 20 Fall in the oxygen tension measured inside an anaerobic jar both above and submerged in the culture medium.

that 12 h undisturbed culture may not be sufficient to achieve anaerobic conditions amongst the cells in culture. Whereas anaerobiasis was on average achieved within 40 minutes in the gas contained within the jar, more than 30 h on average was required to obtain similar conditions at a depth of 8 mm in the stationary medium of such a jar.

Parasite growth in cells containing a haemoglobin of high oxygen affinity

As a further means of testing the possible requirement of the parasite for oxygen, parasites were cultured in cells containing a haemoglobin functionally similar to Hb F in its high affinity for oxygen. Cells containing Hb Radcliffe, described in Chapter 3, were obtained for this purpose. The rates of invasion and development of parasites in such cells were compared to the red cells of normal adults (Table 30). These results were found to be equivocal. In three experiments (1, 2 and 3), there was little difference in either the rates of invasion or of parasite development between the two cell types. However, in two experiments (4 and 5), the rates of invasion were low, whilst in three there was a retardation of parasite growth (4, 5 and 6) when compared to that of normal adults. In one experiment (7), the parasites inside the cells containing the high oxygen affinity haemoglobin failed to grow at all. Although not conclusive, the similar invasion and growth data for these cells as compared to normal adult cells seen in the first three experiments seemed to indicate no protective effect *in vitro* by these cells containing a haemoglobin with a high affinity for oxygen. Deductively,

Experimental details	Expt. No.	1		2		3		4		5		6	
	Donor cells	7662		76113		76115		764		7655		7661	
	Recipient cells	7660	7656	76100	76107	76100	76107	7610	767	7660	7656	7653	7656
	Cell type	C	T	C	T	C	T	C	T	C	T	C	T
Invasion	Parasites (%)	6.1	5.6	7.8	7.1	3.1	3.0	7.8	3.4	< 1		7.9	7.2
	R	/	0.9	/	0.9	/	1.0	/	0.4	/	-	/	0.9
Growth morphology	MGS	3.2	3.3	3.2	3.2	3.2	3.1	3.4	2.9	3.3	2.5	3.7	2.7
	D	/	+0.1	/	0.0	/	-0.1	/	-0.5	/	-0.8	/	-1.0

Note that neither R nor D for individual experiments are consistent

Mean R = 0.8

Mean D = -0.4

Table 30 Invasion and growth of P. falciparum in cells containing haemoglobin with a high oxygen affinity (Hb Radcliffe)

it might therefore appear that the high oxygen affinity of Hb F, in the in vitro situation at least, was not responsible for the retardation of parasite growth seen in cells containing this haemoglobin.

Parasite invasion and growth in fetal haemoglobin containing cells exposed to different oxygen tensions

It was felt that if the retardation of parasite growth in cells containing Hb F was related to the levels of oxygen present, then this effect might be accentuated or diminished by lowering oxygen tensions.

Cultures of donor cells and Hb F-containing recipient cells were grown in microtissue culture plates under three conditions of oxygen tension. The first was 5% CO_2 in air (aerobic), the second was 5% O_2/5% CO_2/in nitrogen (low O_2), whilst the third involved a change in oxygen tensions in an attempt to simulate the in vivo situation. In this last mentioned method, parasites were initially grown aerobically, but were transferred to conditions of 5% O_2/5% CO_2/in nitrogen once the majority of parasites had reached the small trophozoite stage (plunge). This corresponds in vivo to the time of migration of parasites to the deep relatively deoxygenated tissues of the spleen, liver, brain, bone marrow, etc.

The findings (Table 31) can be summarised as follows:-

1) The invasion and growth data under aerobic conditions confirmed the earlier findings, namely of invasion rates commensurate with cell age and retardation of growth in the cord cells compared to normal adult cells (mean R = 1.6, D = -1.6).

Experimental details	Expt. No.	1											
	Donor cells	77128											
	Conditions	Aerobic				Low O_2				Plunge			
	Recipient cells	77125	77126	77123	77124	Same as aerobic							
	Cell type	A	A	F	F	A	A	F	F	A	A	F	F
	Reticulocytes (%)	0.4	0.7	6.2	4.8	Same as aerobic							
	Parasites (%)	12.8 / 11.8	10.8 / 11.8	18.5	22.7	11.8	13.0	13.5	20.5	Same as			
	R	/	/	1.6	1.9	1.0	1.1	0.7	0.9	aerobic			
Growth morphology	MGS	4.2 / 4.1	4.0 / 4.1	2.4	2.3	4.9	4.3	3.4	0.6	4.2	4.0	2.0	2.4
		/	/	-1.7	-1.8	+0.8	+0.2	+1.0	-1.7*	+0.1	0.0	-0.4	+0.1
Isoleucine incorporation	dpm $x10^{-3}$ per well	27.3	13.7	12.8	20.9	54.0	24.3	21.5	41.1	31.5	17.3	10.1	21.0
	dpm $x10^{-3}$ per % parasites	2.1 / 1.7	1.3 / 1.7	0.7	0.9	4.6	1.9	1.6	2.0	2.5	1.6	0.5	0.9
	R incorp	/	/	0.4	0.5	2.2	1.5	2.3	2.2	1.2	1.2	0.7	1.0

* Isoleucine incorporation data does not confirm this result. This value has been omitted in calculation of the summary of these experiments.

Table 31 Invasion and growth of P. falciparum in cord cells under various oxygen tensions

Cont. overleaf

Experimental details	Expt. No.	2											
	Donor cells	77130											
	Conditions	Aerobic				Low O_2				Plunge			
	Recipient cells	77131	77132	77133	77134	Same as aerobic							
	Cell type	A	A	F	F	A	A	F	F	A	A	F	F
	Reticulocytes (%)	0.6	0.7	4.5	5.6	Same as aerobic							
Invasion	Parasites (%)	3.7 3.6 / 3.7		5.2	4.3	4.2	3.7	5.2	5.1	Same as			
	R	/	/	1.4	1.2	1.1	1.0	1.0	1.2	aerobic			
Growth morphology	MGS	3.7 3.5 / 3.6		1.9	2.4	4.6	4.7	3.6	4.2	3.7	3.3	2.3	2.3
	D	/	/	-1.6	-1.2	+1.0	+1.1	+1.7	+1.8	0.0	-2.0	+0.4	-0.1
Isoleucine incorporation	dpm $x10^{-3}$ per well	5.5	2.9	1.8	2.1	14.7	6.2	9.3	7.4	3.9	2.8	2.0	2.3
	dpm $x10^{-3}$ per % parasites	2.1 1.3 / 1.7		0.3	0.5	3.5	1.7	1.8	1.5	1.1	0.8	0.4	0.5
	R incorp	/	/	0.2	0.3	3.5	1.7	1.8	1.5	0.5	0.6	1.3	1.0

Table 31 (cont)

Cont overleaf

		R	D
A	Aerobic	-	-
cells	Low O_2	1.1	+0.8
	Plunge	-	0
F	Aerobic	1.6	-1.6
Cells	Low O_2	1.0	+1.5
	Plunge	-	0

N.B. Low O_2 and plunge experiments - controls are the same cells under aerobic conditions (not Hb A-containing cells).

Table 31 cont Condensed summary of data.

2) In many cases the rates of invasion were higher under low oxygen tensions when compared to their aerobic counterparts (e.g. for A cells R = 1.1). Exceptions to this were the rates of invasion of cord cells in experiment 1.

3) The results also confirmed the observation of improved growth of parasites of P.falciparum under low oxygen tensions, seen previously for parasites placed in anaerobic jars for periods of 12h, but in this experiment placed in 5% oxygen. This applied to both Hb A (mean D = +0.8) and Hb F-containing cells (mean D = +1.5). The morphological data on growth showed a dramatic reduction in parasite development under low tension in one cord blood in experiment 1 (D = -1.7) but this was not confirmed by the incorporation data (R incorp = 2.2). The overall data indicated an improvement in parasite growth regardless of the haemoglobin contained within the particular red cell.

4) Parasite growth however was consistently better in adult cells when compared to the Hb F containing cord cells under the same conditions. The average difference in growth scores between adult and cord cells in each individual set of conditions was of the same order (between -1 and -2).

In conclusion, no clear evidence was obtained to demonstrate an exaggeration or diminution of the retardation of parasite growth in Hb F containing cells under conditions of relative oxygen deprivation. Parasites in both haemoglobin types showed an improvement of parasite growth under lowered oxygen tension with increased rates of invasion in some cultures. This may have been a reflection of improved growth under these conditions giving rise to schizonts with more merozoites either in terms of numbers or viability. Likewise it may reflect improved invasion

of parasites under lowered oxygen tension.

B THE OUTCOME OF A FULL CYCLE OF PARASITE GROWTH IN CELLS CONTAINING Hb F

Although this system of parasite culture in tissue culture trays did not usually allow adequate growth of a third generation of parasites in vitro, in one experiment it was possible to count the third generation of ring forms. This was after a cycle in either Hb A-containing normal adult cells, or Hb F-containing cord cells. These cultures were carried out in parallel.

The results of this experiment are shown in Table 32. Despite the fact that the cord cells had shown a higher rate of invasion in the second generation (R = 1.7) there was a reversal in parasite multiplication in the third generation with far fewer parasites present in the cultures containing Hb F-containing cells (R = 0.5). It should be noted that the numbers of parasites involved in this third generation are small. Nevertheless, this striking reversal of parasite numbers in the two cultures, after a full cycle of parasite growth in the particular cell type, might imply that the parasites had not grown well in the Hb F-containing cells as compared to the Hb A-containing cells. The outcome of this was to give rise to fewer merozoites, which led to a reduced rate of invasion of red cells in the third generation in vitro.

C EVIDENCE FOR HUMORAL ANTI-PARASITIC ACTIVITY IN THE SERUM OF NEWBORNS OF MOTHERS LIVING IN AN ENDEMIC AREA FOR MALARIA

Of particular interest was the presence or absence of

Blood type		Adult			Cord				
Donor cells		7639							
Recipient cells		7647	7645	7646	7640	7642	7643	7644	7641
2nd generation ring forms (%)		5.8	4.4	4.0	7.7	7.7	8.1	6.6	9.7
3rd generation	Total cells counted	1052	1080	1098	1058	1114	1174	1170	1089
	Parasites counted	38	26	31	18	27	13	16	8
	Parasites (%)	3.6	2.4	2.8	1.7	2.4	1.1	1.4	0.7

Mean R (second generation) = 1.7

(third generation) = 0.5

Table 32 Third generation ring forms of P. falciparum arising after a cycle of development in vitro in either Hb A or Hb F-containing cells.

humoral immunity directed against P. falciparum in the serum of newborns in an area endemic for malaria. This was because it still remains unclear whether passively-transferred transplacental immunity is sufficient to account for the low incidence and mortality from malaria seen during the first few months of life.

The test sera came from the umbilical cords of normal newborn Gambians born of mothers at the Royal Victoria Hospital, Banjul, the Gambia, and who were known not to have taken anti-malarials during late pregnancy. The control sera were taken from the umbilical cords of four newborns born at the John Radcliffe Hospital, Oxford, U.K. The mothers of these children had not lived in a malarious area, nor had they taken any malaria prophylaxis in the past. They could thus be considered non-immune with regard to malaria. The technique used was that of Wilson and Phillips (1976). Donor and recipient cell suspensions were made up with supplemented Medium 199 and 10 mM HEPES but without AB serum and 150 µl of the suspension was placed in each culture well. The recipient cells were cord cells from the umbilical cords of normal Gambian newborns. To each well 100 µl of the test or control sera was added, i.e. 2/5 by volume. After invasion of the recipient cells had occurred, blood smears prepared from duplicate wells were counted for parasites. The results of four experiments using different donor bloods in each case are shown in Table 33.

It can be seen that on average the rates of multiplication of parasites are lower in the presence of Gambian cord sera, but that in certain cases (particularly

Expt. No.		1	2	3	4
Donor cells		7741	7779	7790	7798
Recipient cells		7719	7788	7788	7788
Oxford	C 1	12.5	7.6	6.1	11.0
	C 2	17.2	8.7	7.5	10.3
	C 3	10.3	9.5	4.8	6.7
	C 4	8.9	4.1	2.2	8.0
	Mean ± SD	12.2 ±3.6	7.5 ±2.4	5.2 ±2.3	9.0 ±2.0
Gambian	C 1	8.9	4.3	2.9	1.6
	C 2	7.1	1.9	1.2	0.8
	C 3	3.6	4.7	0	-
	C 4	12.5	8.9	0.4	3.7
	C 5	3.3	1.8	2.7	6.8
	C 6	-	13.3	3.8	6.9
	C 7	-	9.2	2.6	3.0
	C 8	-	3.1	0.6	9.8
	C 9	-	1.3	1.1	0
	C 10	-	0.3	1.6	2.5
	C 11	-	13.7	0	0.9
	C 12	-	7.5	2.1	6.3
	Mean ± SD	7.1 ±3.8	5.8 ±4.4	1.6 ±1.2	3.8 ±3.0
	P	>0.05	>0.10	<0.01	<0.01

Table 33 Invasion by P. falciparum of red cells in the presence of serum obtained from the umbilical cords of newborns.

experiment 2) there were Gambian sera which showed no decrease in the invasion rates when compared to the controls. Moreover those Gambian sera which allowed good invasion with one donor blood did not necessarily sustain this effect with the others and vice versa. Inhibition of invasion by Gambian serum appeared unpredictable when using randomly selected parasites. The invasion in duplicate cultures with the same source of parasites were however comparable, showing that the ability of a serum to inhibit in one experiment, but not another, was unlikely to be an artefact of the system.

In view of the large range of rates of invasion seen in the Gambian cord sera, it seemed inappropriate to apply statistical tests. However, using the Student's t test, it was found that the reduction in invasion seen was only statistically significant in two of the four experiments. The results therefore suggested that, at birth at least, there was a factor present in the serum of newborn Gambians which diminished the number of falciparum parasites invading uninfected red cells in culture compared to serum from newborns born in Oxford. However this was not consistent, especially when different donor bloods were used.

The experiments carried out in this section were not conclusive. The two most likely mechanisms by which Hb F retards parasite growth were relative oxygen deprivation or the relative inability of the parasite to degrade this haemoglobin. It appeared that the presence of a minimal amount of oxygen was necessary in order to

culture malaria parasites, but whether this was specifically a parasite requirement, was not established. Under low oxygen tensions, growth was in fact enhanced, whereas in the absence of oxygen parasites were unable to grow. The reason for this remains unknown. In cells containing a haemoglobin of high oxygen affinity, parasites were certainly capable in some cases of invading and growing under aerobic conditions as well as in normal adult cells. No alteration in the degree of growth retardation could be detected in Hb F-containing cells when exposed to low oxygen tensions. However, after a single cycle inside Hb F-containing cord cells, parasites gave rise to fewer small rings than in normal adult cells. Data were also presented to cast some doubt on the efficacy of passively-transferred immunity from the mother in protecting a newborn from malaria. These latter data emphasised the possible importance of Hb F in protection of certain individuals against malaria, but the exact mechanism whereby Hb F retards parasite growth could not be determined.

CHAPTER 7

P. FALCIPARUM AND OTHER RED CELL DISORDERS

The in vitro culture system was also used to assess the invasion and growth of P. falciparum in the red cells of other haemoglobinopathies and red cell disorders.

β thalassaemia trait

Samples of blood from three carriers of β thalassaemia were tested in seven experiments (Table 34). The rates of invasion were marginally higher in the thalassaemic blood when compared with normal adult controls (mean R = 1.2), which is in keeping with the slightly shortened red cell survival seen in this condition. However, there was no evidence at all of retardation of parasite growth in the thalassaemic cells (mean D = +0.1). Experiment No. 7 in particular showed that parasites were quite capable of attaining the mature schizont stage in these microcytic hypochromic cells (Appendix C). Parasite growth in a typical culture with β thalassaemia trait is shown in Plate XVII.

Iron deficiency anaemia

Blood was obtained in the U.K. from a patient with iron deficiency anaemia due to chronic gastro-intestinal bleeding. These cells were examined for invasion and growth of parasites particularly to see if their small size or under-haemoglobinisation rather than their need for free iron had any effect on the invasion or growth of parasites

Experimental details	Expt. No	1		2		3		4		5		6		7	
	Donor cells	7655		76113		764		7662		76115		76112		7661	
	Recipient cells	7660	7657	76100	76108	7610	768	7660	7657	76100	76108	76100	76108	7653	7657
	Cell type	C	T	C	T	C	T	C	T	C	T	C	T	C	T
Invasion	Parasites (%)	< 1		7.8	6.8	7.8	7.8	6.1	6.7	3.1	3.6	6.2	8.4	7.9	10.8
	R	/	/	/	0.9	/	1.0	/	1.1	/	1.2	/	1.4	/	1.4
Growth morphology	MGS	3.3	2.9	3.2	3.3	3.4	3.5	3.4	3.5	3.2	3.4	3.6	3.8	3.7	4.1
	D	/	-0.4	/	+0.1	/	+0.1	/	+0.1	/	+0.2	/	+0.2	/	+0.4

Mean R = 1.2

Mean D = +0.1

Table 34 Invasion and growth of P. falciparum in cells of β thalassaemia trait.

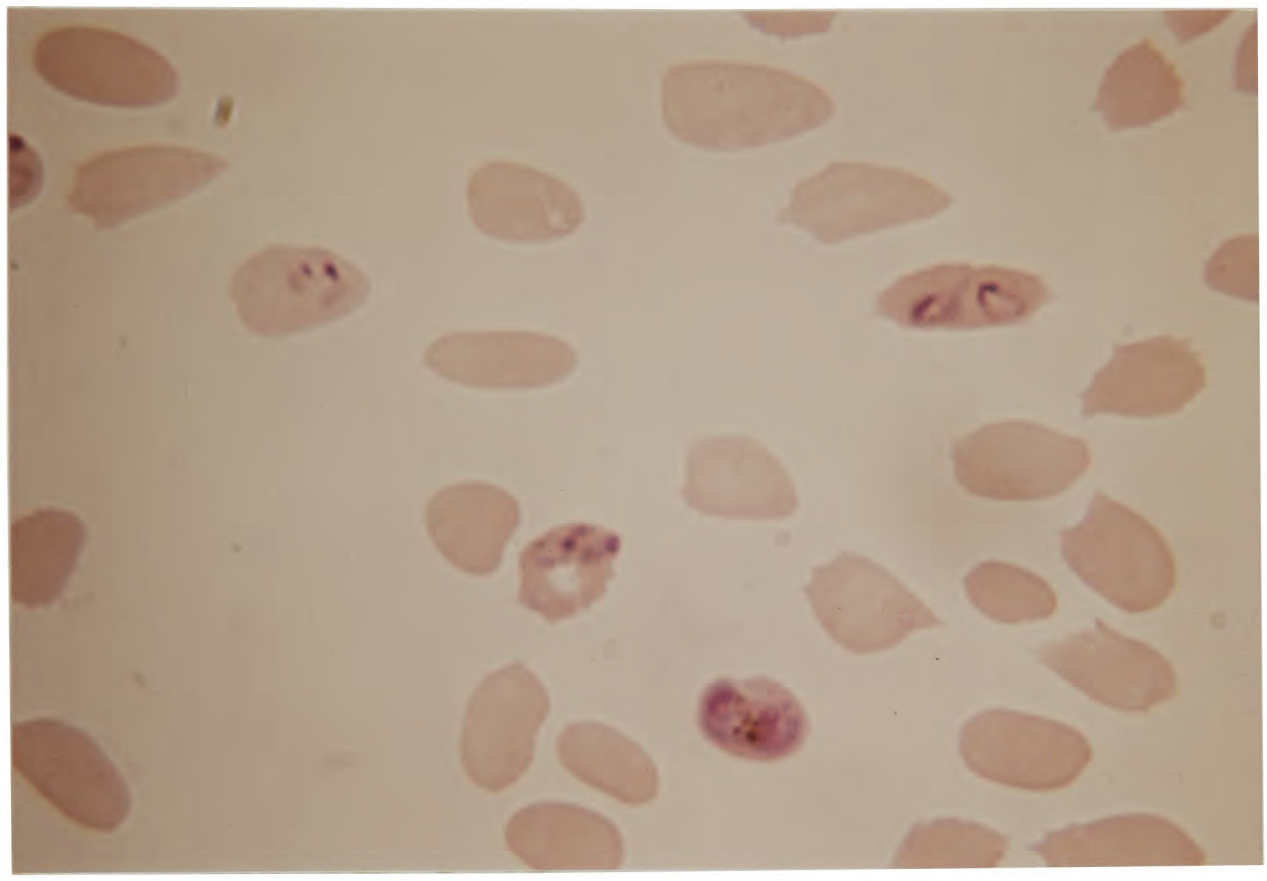


PLATE XVII Growth of P. falciparum in the cells of thalassaemia trait (x 1500).

inside them. Medium 199 itself contains only 0.1 mg per litre of iron as ferric nitrate (Appendix A). In the first experiment, the percentage parasites present after invasion was insufficient to compare the two types of cells. However, in the second, a slightly higher rate of invasion was seen in the cells of the iron deficient individual (Table 35). When growth of parasites was assessed in the two experiments, a slight retardation was seen in one, with improved growth in the other when compared to the controls. There was certainly no evidence for any pronounced retardation in growth.

Sickling disorders

Red cells from individuals both heterozygous (AS) and homozygous (SS) for Hb S were examined for the rates of invasion and subsequent growth of parasites in them, and compared to normal adult (AA) cells. Both aerobic (5% CO_2 in air) and low oxygen tensions (5% O_2) were used in culture. Cultures carried out at low oxygen tensions were also fixed in suspension by adding 50 µl of 40% buffered formaldehyde (pH 7.4) at $4^{o}C$ to the appropriate wells immediately after removal from the gassed environment. This was done in order to fix those cells which had sickled *in vitro*. Thus, the distribution of parasites amongst the cells which had actually sickled and those which had not could be examined. Such smears were unfortunately not suitable for detailed assessment of parasite morphology (Plate XVIII).

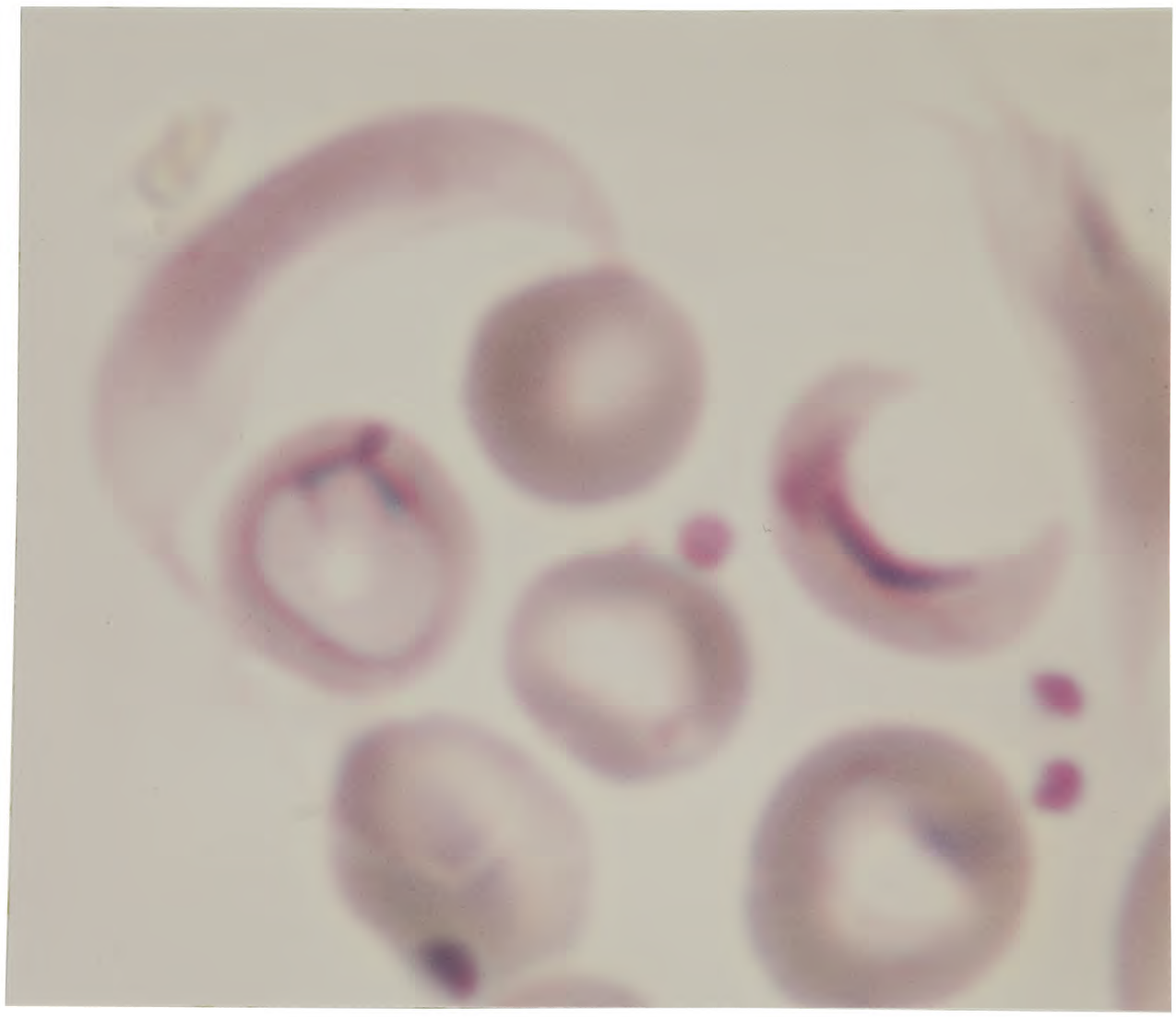


PLATE XVIII Distribution of P. falciparum in blood from a homozygous sickler showing parasites in both a sickled and non-sickled cell (x 3000).

Experimental details	Expt. No	1		2	
	Donor cells	7655		7662	
	Recipient cells	7660	7658	7660	7658
	Cell type	C	T	C	T
Invasion	Parasites (%)	< 1		6.1	6.7
	R	/	/	/	1.1
Growth	MGS	3.3	3.1	3.2	3.4
	D	-	-0.2	-	+0.2

Table 35 Invasion and growth of P. falciparum in cells from a patient with iron deficiency anaemia.

Invasion and development of parasites in cells from sickle heterozygotes (AS) Under aerobic conditions the rate of invasion into AS cells was similar to that of normal adult cells (mean R = 1.0) (Table 36a). Growth in the AS cells showed only slight retardation overall (mean D = -0.1) under these aerobic conditions. However, under low oxygen tensions significant differences emerged. There was a reduction in the rate of invasion of parasites into AS cells when compared to their aerobic counterparts (mean R = 0.8), whilst in the AA cells the rates of invasion increased (Table 36b) (mean R = 1.1). Using the Students 't' test the difference between the rates of invasion of AS and AA cells under these conditions was statistically significant ($0.02 > p > 0.01$). Moreover, formaldehyde fixed smears revealed that very few of the cells had sickled in the AS cells under the reduced oxygen tensions and that the majority of parasites were in the non-sickled cells. Thus, the observed decreased rate of invasion was not restricted to the sickled forms only but these decreased invasion rates applied to the non-sickled cells as well.

Growth of parasites under low oxygen tensions in the AS cells was even more strikingly affected (Table 36b) (Fig. 21). Marked retardation was seen in all cultures when compared to parasites in AS cells under aerobic conditions (mean D = -2.4), and in a few cases all parasites appeared abnormal, indicating inhibition of growth. In distinct contrast, control cultures in AA cells showed improved growth under similar conditions (mean D = +0.2). Even at this late stage in the cultures, formaldehyde fixed smears showed that the majority of parasites were present in non-sickled cells, and this indicated that sickling was not itself necessary

Experimental details	Expt. No.	1			2			3			4			5		
	Donor cells	76136			7796			7798			7740			7746		
	Recipient cells	77126	77127	77128	7789	7748	7747	7789	7748	7747	7744	7748	7747	7744	7748	7747
	Cell type	AA	AS	AS	AA	AS	AS	AA	AS	AS	AA	AS	AS	AA	AS	AS
	Reticulocytes (%)	0.8	1.0	1.2	1.0	1.1	1.3	1.0	1.1	1.3	2.5	1.1	1.3	2.5	1.1	1.3
Invasion	Parasites (%)	1.7	1.2	1.5	15.5	13.5	16.5	12.1	10.6	12.8	10.3	13.4	14.6	7.7	8.1	8.6
	R	/	0.7	0.9	/	0.9	1.1	/	0.9	1.1	/	1.3	1.4	/	1.1	1.1
Growth morphology	MGS	2.7	3.0	2.8	3.9	3.8	3.6	5.3	5.4	5.3	2.8	2.9	2.2	2.4	2.2	2.1
	D	/	+0.3	+0.1	/	-0.1	-0.3	/	+0.1	0.0	/	+0.1	-0.6	/	-0.2	-0.3

Mean R = 1.0

Mean D = -0.1

Table 36a Invasion and growth of P. falciparum in cells from sickle heterozygotes (AS) under aerobic conditions.

(Cont over)

Experimental details	Expt. No	6				7		8		9		10 (Growth at 42 h)			10 (Growth at 54 h)
	Donor cells	7790				77104		77109		77113		77128			77128
	Recipient cells	7744	7789	7748	7747	77108	7747	77108	7747	77108	7747	77125	77126	77127	Same as at 42 h
	Cell type	AA	AA	AS	AS	AA	AS	AA	AS	AA	AS	AA	AA	AS	
	Reticulocytes (%)	2.5	1.0	1.1	1.3	1.0	1.1	1.0	1.1	1.0	1.1	0.4	0.7	1.8	
Invasion	Parasites (%)	$\frac{5.9 \quad 6.2}{6.1}$		6.4	6.1	10.3	8.2	18.7	17.2	7.7	6.8	$\frac{12.8 \quad 10.8}{11.8}$		15.5	
	R	/	/	1.0	1.0	/	0.8	/	0.9	/	0.9	/	/	1.3	
Growth morphology	MGS	$\frac{3.1 \quad 3.0}{3.1}$		2.9	2.6	3.2	2.8	4.8	4.6	2.9	2.8	$\frac{3.2 \quad 2.8}{3.0}$		3.0	$\frac{4.2 \quad 4.0}{4.1}$ 4.1
	D	/	/	-0.2	-0.5	/	-0.4	/	-0.2	/	-0.1	/	/	0.0	/ / 0.0

Table 36a (cont)

Experimental details (correspond to Table 36a)	Expt. No.	1*			2				4		6			8		9		10 (42 h)			10 (54 h)		
	Cell type	AA	AS	AS	AA	AS	AS	AA	AS	AS	AA	AS	AS	AA	AS	AA	AS	AA	AA	AS	AA	AA	AS
Invasion	Sickled cell %	0	42	14	0	2	7	0	0	1	0	2	3	0	3	0	1	0	0	5	Same as at 42 h		
	Parasites %	1.5	0.7	0.6	14.9	12.4	13.2	10.5	12.7	13.4	6.9	6.1	5.5	18.3	18.9	12.2	4.3	11.8	13.0	12.2			
	R	0.9	0.6	0.4	1.0	0.9	0.8	1.0	0.9	0.9	1.6	0.9	0.9	1.0	1.1	1.3	0.6	0.9	1.2	0.8			
Growth	Sickled cells %	0	66	48	0	6	24	0	3	7	0	7	24	0	6	0	8	0	0	5	0	0	8
	MGS	2.9	0	0	4.1	0.3	0.6	1.7	0.9	0.3	4.1	1.0	0.4	5.2	0	3.4	2.4	3.2	2.7	0.1	4.9	4.0	0.1
	D	+0.2	-3.0	-2.8	+0.2	-3.5	-3.0	-0.9	-2.0	-1.9	+1.0	-1.9	-2.2	+0.4	-4.6	+0.5	-0.4	0.0	-0.1	-2.9	+0.7	0.0	-4.0

*This experiment done in 1976 was in an anaerobic jar - the others are at an oxygen tension of 5%

Mean R (AA) = 1.1
(AS) = 0.8

Mean D (AA) = +0.2
(AS) = -2.4

Table 36b Invasion and growth of P. falciparum in cells from sickle heterozygotes (AS) under low oxygen tensions

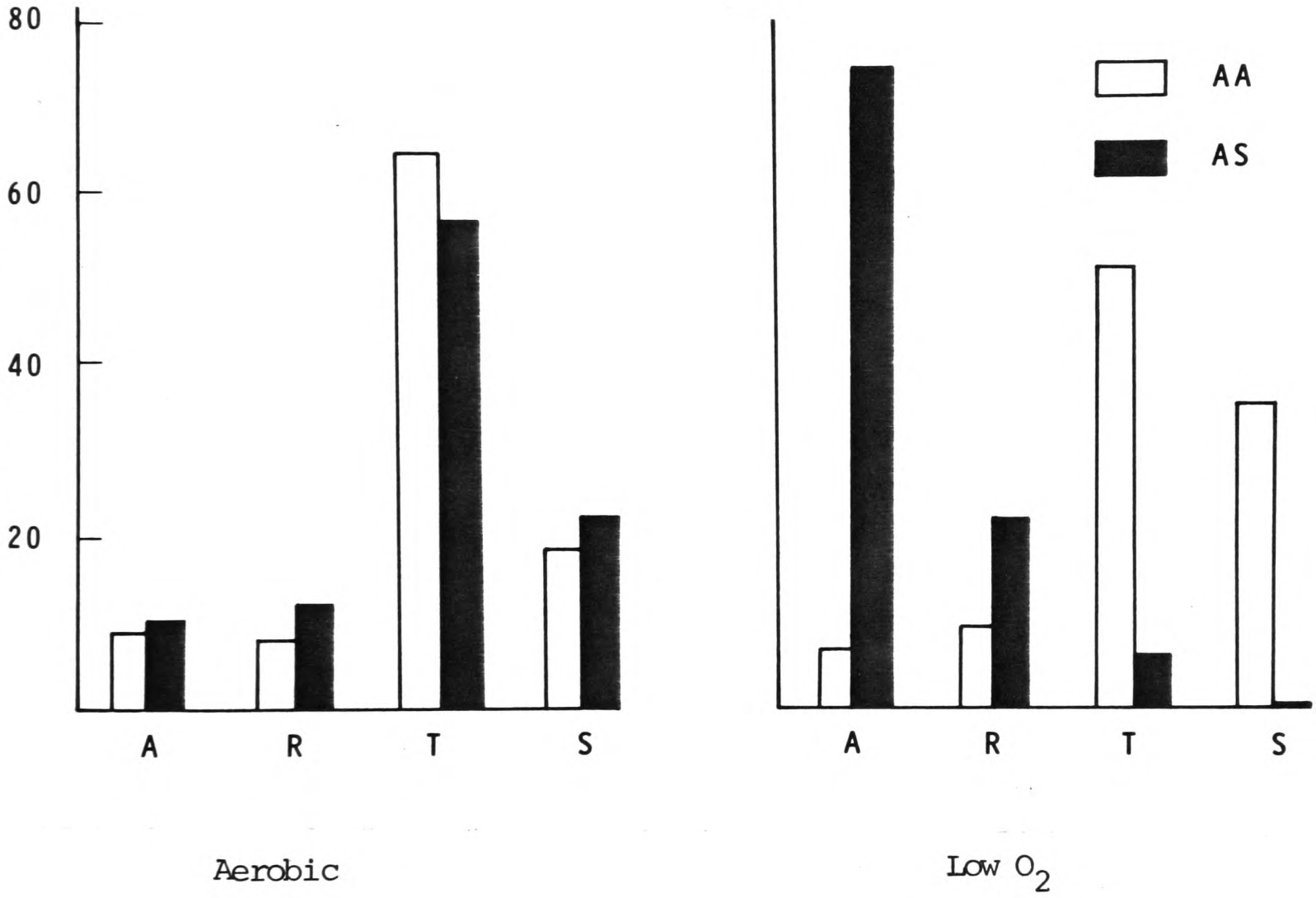


Figure 21 Growth of P. falciparum in cells from sickle heterozygotes (AS) under aerobic coditions and low oxygen tensions as compared to normal adult controls (AA).

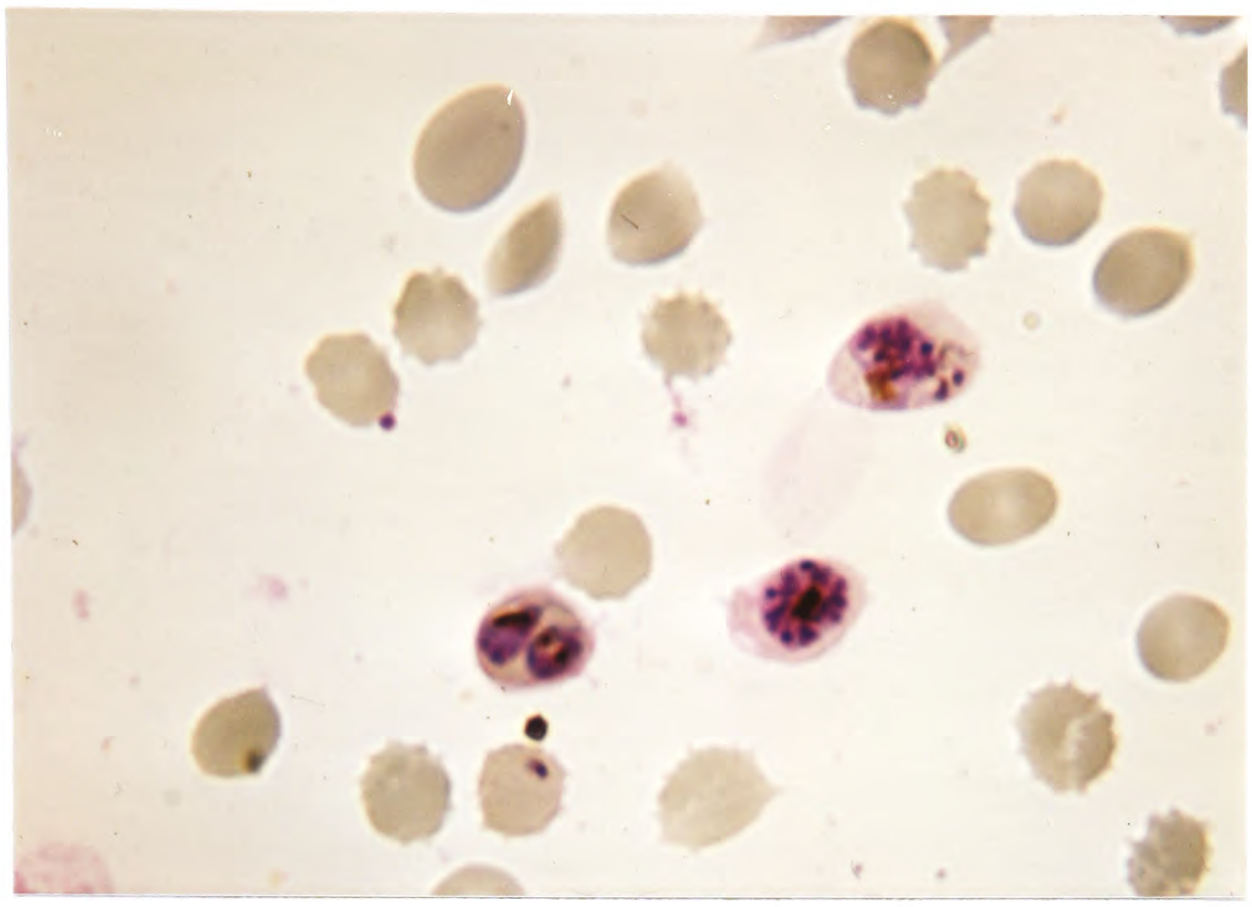
for growth retardation of parasites in AS cells under low oxygen tension. (Plate XIX a, b and c).

Invasion and development of parasites in cells from sickle homozygotes (SS) In the SS cells, the findings were similar to those seen in the heterozygotes, but a significant increase in invasion was seen in them under aerobic conditions (mean R = 1.3) when compared to AA cells (Table 37a). This was presumed to reflect the fact that the SS cells are relatively young and might therefore have higher rates of invasion. Growth in the SS cells was only slightly retarded under aerobic conditions (mean D = -0.3).

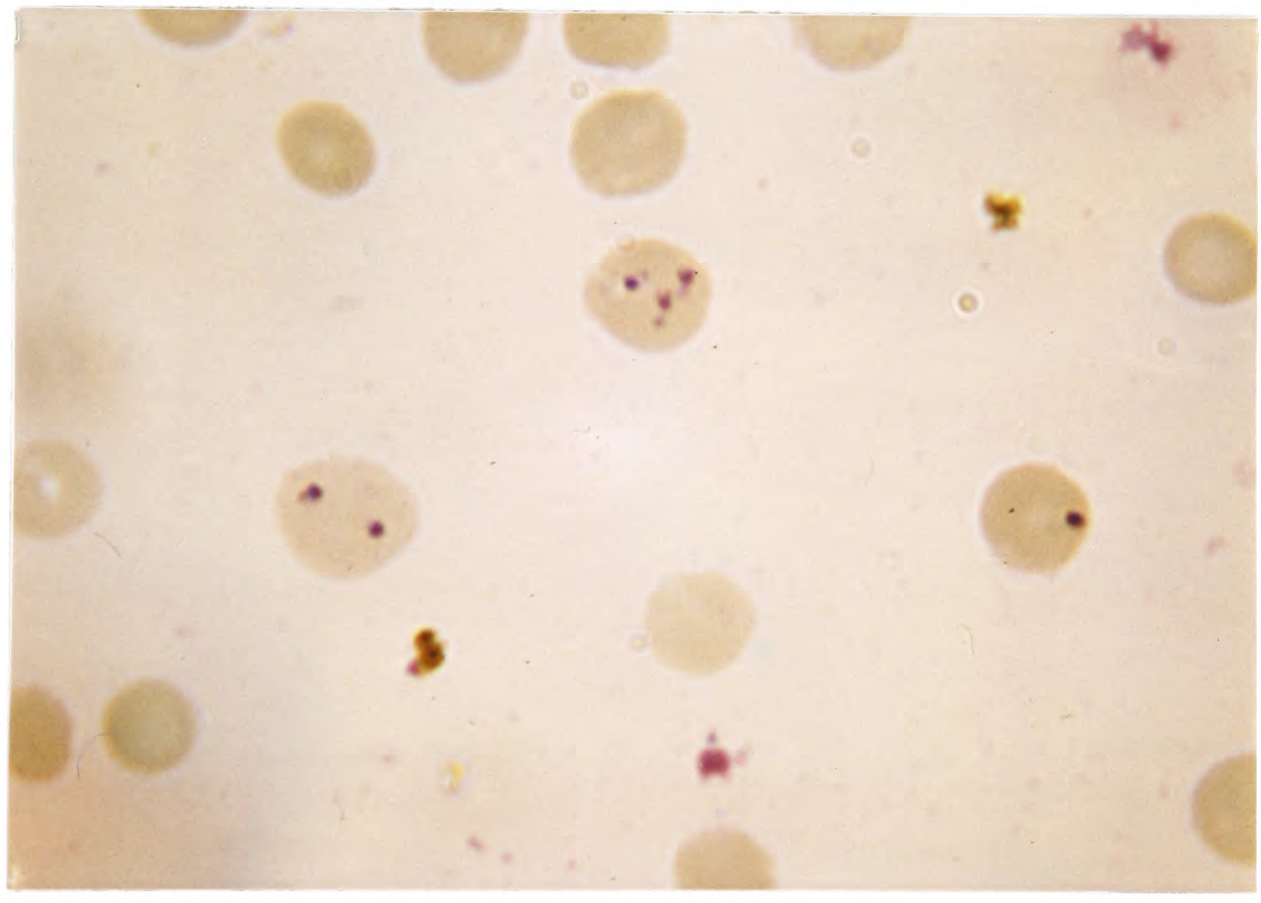
However, the reduction in invasion in SS cells was even more striking than in AS cells when the oxygen tension was lowered. There was a significant decrease in the invasion rate in SS cells when compared to their aerobic counterparts (mean R = 0.6), whereas the rate of invasion in the AA cells was similar under low oxygen tension as under aerobic conditions (mean D = 1.0). Using the Students't' test the differences between AA and SS cells under these conditions were significant ($0.05 > p > 0.02$). (Table 37b).

Growth retardation in SS cells under low oxygen tension was of a similar order to AS cells (mean D = -2.4). Once again, an improvement in parasite growth in control AA cells was seen under reduced oxygen tensions (mean D = +0.4).

Formaldehyde fixed smears in SS cells under low oxygen tensions indicated that the reduction in invasion had occurred to a similar degree in both sickled and non-sickled cells. A typical example of the distribution of parasites in such a preparation in shown in Table 37c, Plate XVIII.



a) Aerobic culture. Note mature parasites



b) Low oxygen tension. No parasites have developed.

PLATE XIX Growth of P. falciparum in blood from a heterozygous sickler (AS).

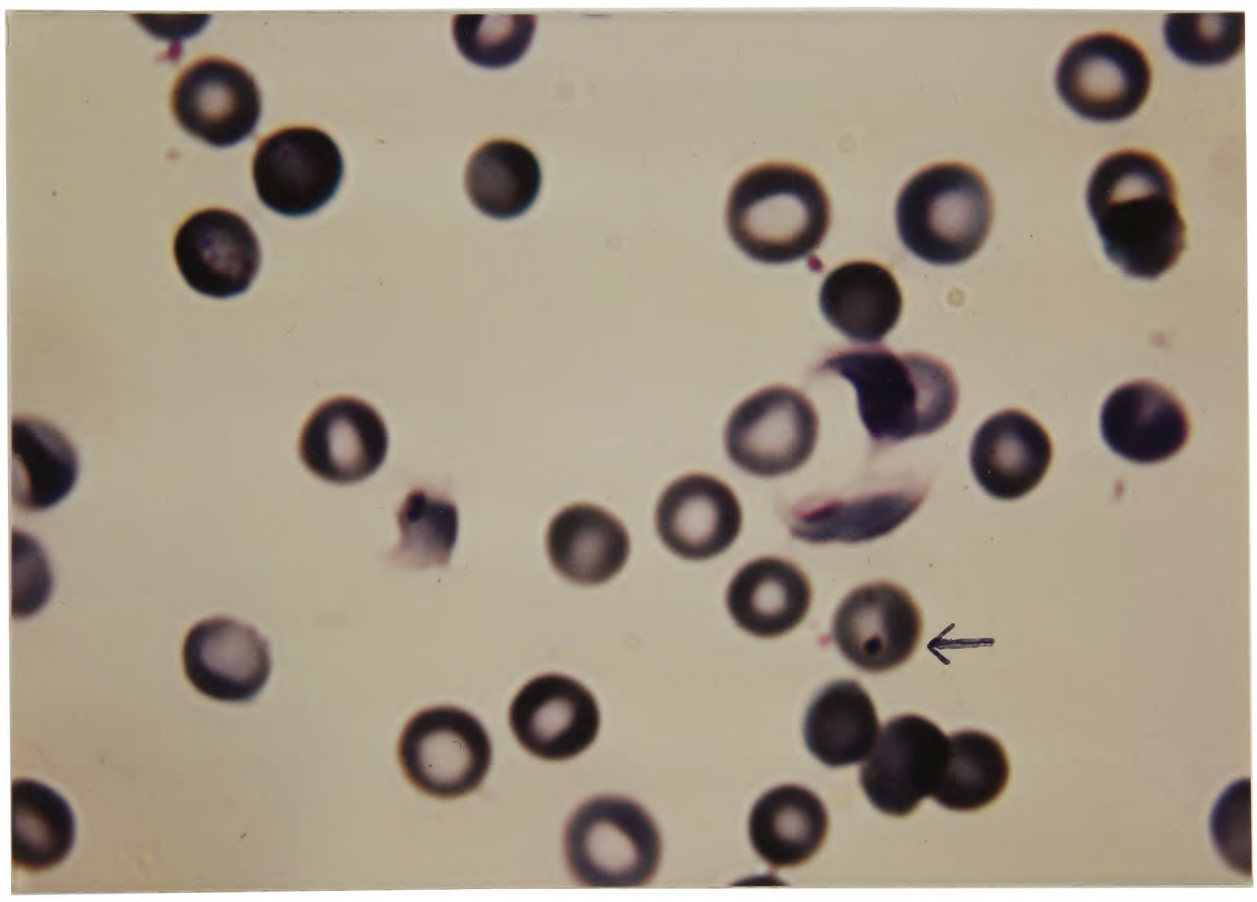


PLATE XIX cont. c) Low oxygen tension and fixed in formaldehyde. Arrow indicates parasite in non-sickled cell. Only very few of the cells have sickled.

Experimental details	Expt. No	1		2			3		4		5	
	Donor cells	7686		76136			77113		77118		77119	
	Recipient cells	7683	7682	76126	76129	76137	77108	77112	77111	77112	77111	77112
	Cell type	AA	SS	AA	SS	SS	AA	SS	AA	SS	AA	SS
	Reticulocytes	1.3	5.8	0.8	20.0	18.0	1.2	15.0	1.2	15.0	1.2	15.0
	Hb F %	<4	29.2	<4	-	-	<4	9.5	<4	9.5	<4	9.5
Invasion	Parasites %	16.8	22.9	1.7	2.1	2.0	10.4	12.9	18.5	21.2	10.9	14.9
	R	/	1.4	/	1.2	1.2	/	1.2	/	1.2	/	1.4
Growth morphology	MGS	3.0	2.8	2.7	2.3	2.4	2.8	2.5	4.2	3.7	4.8	4.6
	D	/	-0.2	/	-0.4	-0.3	/	-0.3	/	-0.5	/	-0.2

Mean R = 1.3

Mean D = -0.3

Table 37a Invasion and growth of P. falciparum in cells from sickle homozygotes (SS) under aerobic conditions

Experimental details (correspond to Table 37a)	Expt. No.	3		4		5		1*		2*		
	Cell type	AA	SS	AA	SS	AA	SS	AA	SS	AA	SS	SS
Invasion	Sickled cells (%)	0	55	0	50	0	98	0	54	0	86	89
	Parasites (%)	12.2	11.4	16.3	14.4	14.6	9.5	15.4	11.6	1.5	0.7	0.9
	R	1.2	0.9	0.9	0.7	1.3	0.6	0.9	0.5	0.9	0.3	0.5
Growth morphology	Sickled cells (%)	0	89	0	94	0	99					
	MGS	3.3	1.2	4.3	1.0	5.4	1.4					
	D	+0.5	-1.3	+0.1	-2.7	+0.6	-3.2					

* These two experiments done in 1976 were under anaerobic conditions, the others are at an oxygen tension of 5%

Mean R (AA) = 1.0 Mean D (AA) = +0.4

R (SS) = 0.6 D (SS) = -2.4

Table 37b Invasion and growth of P. falciparum in cells from sickle homozygotes (SS) under low oxygen tensions.

Cells	Conditions		Cells counted	Parasites counted	Parasites (%)
AA	Aerobic		1166	180	15.4
SS	Aerobic		1921	440	22.9
	Low oxygen tensions	Sickled cells (51%)	1912	199	10.4
		Non-sickled cells (49%)	1802	208	11.5

Table 37c Distribution of P. falciparum in sickled and non-sickled cells of s sickle homozygote (SS) where invasion has taken place under low oxygen tensions.

where about 50% of the cells had sickled when the counts were done. Parasitisation of sickled and non-sickled cells was 10.4 and 11% respectively but each was very much lower than the rates of invasion of SS cells under aerobic conditions (22.9%). This indicated that the process of sickling was not a prerequisite to bring about a decrease in the invasion rate under low oxygen tensions. Unfortunately, the effect of sickling on parasite growth in SS cells could not be examined, as almost all the cells had sickled in the cultures at the time of assessment of growth.

Development of parasites in cells containing Hb S at low oxygen tension after an initial period of growth under aerobic conditions One further type of experiment was carried out with AS cells in an attempt to simulate *in vivo* conditions and to see whether reduced oxygen tensions had any effect on the more mature forms of *P. falciparum*. Parasites were cultured to the small trophozoite stage, which corresponds to the time *in vivo* when they would be present in well-oxygenated peripheral blood, and then placed into conditions of low oxygen tension which *in vivo* would correspond to the period when the parasites had moved into the poorly oxygenated tissues of the spleen, liver, bone marrow and brain, etc. After transfer from aerobic conditions to a period of 12 h incubation at low oxygen tension (plunge), blood smears were made and the parasite growth under these conditions was compared to that in controls using the same cells but maintained under aerobic conditions (Table 38). Whereas no change was seen in the AA cells (mean D = 0.0), growth of these more mature parasites was clearly retarded in the AS cells (mean D = -0.9), although not as markedly

Experimental details (correspond to table 36a)	Expt. No	2 (35-47 h)			10 (42-54 h)		
	Cell types	AA	AS	AS	AA	AA	AS
Growth morphology	Sickled cells (%)	0	2	7	0	0	2
	MGS plunge	3.9	3.4	2.5	4.2	4.0	3.0
	MGS aerobic	3.9	3.8	3.6	4.2	4.0	4.1
	D	0	-0.4	-1.1	0	0	-1.1
	MGS low O_2	4.1	0.3	0.1	4.9	4.0	0.1

Mean D (AA) = 0 Mean D (AS) = -0.9

Table 38 Growth of P. falciparum in cells from sickle heterozygotes (AS) which, after growth to small trophozoites under aerobic conditions, have been placed in conditions of low oxygen tension for 12 h. (Plunge)

as in the AS cells which had been maintained as controls in low oxygen tensions (low O_2) from the beginning of the experiment (mean D = -3.7). Once again, examination of formaldehyde-fixed smears showed that very few of the cells had sickled.

From all these experiments involving Hb S, it was concluded that this haemoglobin decreases the rate of invasion and markedly retards the growth of P. falciparum in cells from both heterozygotes and homozygotes but that this effect is dependant on conditions of low oxygen tension. Such conditions need not cause actual sickling of red cells, as it has been shown that these effects described do not require that the cells involved should sickle. Furthermore the growth of mature as well as immature forms of P. falciparum was found to be retarded by Hb S.

Glucose-6-phosphate dehydrogenase (G6PD) deficiency

Preliminary experiments on invasion and development of parasites were carried out on the cells obtained from G6PD-deficient individuals. These included a hemizygous male and heterozygous and homozygous females. The data on parasite invasion showed an increase when compared to normal adult cells (mean R = 1.3) (Table 39). This may reflect a younger mean age of this cell population but this was not borne out by the reticulocyte counts which showed no evidence of active haemolysis in the G6PD-deficient cells (Table 39).

The growth data showed both by morphological and radioisotope incorporation that parasite development was retarded in cells taken from the deficient individuals

Experimental details	Expt. No	1			2			3			4		5	
	Donor cells	7740			7746			7790			766		767	
	Recipient cells	7744	7742	7743	7744	7742	7743	7744	7742	7743	7631	7630	7633	7634
	Reticulocytes	1.5	2.8	1.4	1.5	2.8	1.4	1.5	2.8	1.4	1.6	2.0	1.2	1.8
	G6PD activity*	N	0.56	O	N	0.56	O	N	0.56	O	N	0.57	N	O
	Cell type	C	T	T	C	T	T	C	T	T	C	T	C	T
Invasion	Parasites (%)	10.3	15.0	14.0	7.7	8.2	8.6	5.9	7.0	7.0	8.1	8.8	< 1	
	R	-	1.5	1.4	-	1.1	1.1	-	1.2	1.2	-	1.1	-	-
Growth morphology	Abnormal forms (%)	14	24	22	24	29	46	13	17	41	7	13	10	31
	MGS	2.8	2.4	2.3	2.4	1.9	1.1	3.1	2.8	2.0	3.0	2.7	3.5	2.3
	D	-	-0.4	-0.5	-	-0.5	-1.3	-	-0.3	-1.1	-	-0.3	-	-1.2
Isoleucine incorporation	dpm $x10^{-3}$ per well	17.0	17.8	15.4	8.7	7.0	7.2	9.3	7.7	7.2	30.5	28.5	-	-
	dpm $x10^{-3}$ per % parasites	1.7	1.2	1.1	1.1	0.9	0.3	1.6	1.1	1.0	3.8	3.2	-	-
	R incorp	-	0.7	0.6	-	0.8	0.7	-	0.7	0.6	-	0.8	-	-

* Normal (N) G6PD activity 1.20 - 1.72 enzyme units per 10^{10} RBC at $25^{o}C$

Mean R = 1.2 Mean D = -0.7 Mean R incorp = 0.7

Table 39 Invasion and growth of P. falciparum in cells deficient in glucose-6-phosphate dehydrogenase (G6PD)

(mean D = -0.7, mean R incorporation = 0.7). The morphological data also seemed to indicate that this effect was related to the amount of G6PD activity present. Thus, the difference in growth scores (D) in cultures containing cells from individuals who were totally deficient (mean D = -1.0) was greater than that in cultures with cells from individuals who were only partially deficient (mean D = -0.4).

However, these results must be interpreted with caution as in all experiments the percentage of abnormal parasites even in the controls is high (reaching about 24% - see Appendix C). Moreover these bloods were stored at $4^{o}C$ for at least a week before use and enzyme-deficient cells might well be more labile to metabolic change than those cells containing haemoglobin variants.

Haemoglobin C and E traits

The rates of invasion and subsequent growth of parasites in cells obtained from individuals heterozygous for Hbs C and E are shown in Tables 40 and 41 respectively. For both types of haemoglobin, no difference in invasion rates could be seen when compared to normal adult cells (mean D = 1.0 for both). Similarly, growth of parasites in cells with these haemoglobins showed no significant difference from that in cells containing Hb A (mean D = 0.0, and +0.1 respectively). Cells from individuals homozygous for these conditions were not tested.

Experimental details	Expt. No.	1		2		3			4		
	Donor cells	77109		77113		77118			77119		
	Recipient cells	77108	77105	77111	77105	77108	77111	77105	77108	77111	77105
	Reticulocytes	1.0	1.2	0.5	1.2	1.0	0.5	1.2	1.0	0.5	1.2
	Cell type	AA	AC	AA	AC	AA	AA	AC	AA	AA	AC
Invasion	Parasites (%)	18.7	19.1	10.4	11.6	$\frac{16.1 \quad 18.5}{17.3}$		17.6	$\frac{10.1 \quad 10.9}{10.5}$		11.0
	R	-	1.0	-	1.1	-	-	1.0	-	-	1.0
Growth morphology	MGS	5.2	5.1	3.0	2.9	$\frac{4.2 \quad 4.2}{4.2}$		4.1	$\frac{4.8 \quad 4.7}{4.8}$		5.0
	D	-	-0.1	-	-0.1	-	-	-0.1	-	-	+0.2

Mean R = 1.0 Mean D = 0.0

Table 40 Invasion and growth of P. falciparum in cells from a Hb C heterozygote (AC)

Experimental details	Expt. No.	1				2				3				4		
	Donor cells	77113				77118				77119				77109		
	Recipient cells	77108	77111	77106	77107	Same as in Experiment 1								77108	77106	77107
	Reticulocytes (%)	1.1	0.5	2.4	0.8	"	"	"	"	"	"	"	"	1.0	2.4	0.8
	Cell type	AA	AA	AE	AE	AA	AA	AE	AE	AA	AA	AE	AE	AA	AE	AE
Invasion	Parasites (%)	7.7 10.4 / 9.1		9.1	10.1	16.1 18.5 / 17.3		13.0	16.4	10.1 10.9 / 10.5		8.3	10.6	18.7	17.8	19.3
	R	-	-	1.0	1.1	-	-	0.8	0.9	-	-	0.8	1.0	-	1.0	1.1
Growth morphology	MGS	2.8 2.7 / 2.8		3.0	2.7	4.2 4.2 / 4.2		4.1	4.2	4.8 4.7 / 4.8		5.1	4.7	4.8	4.8	4.9
	D	-	-	+0.2	+0.1	-	-	-0.1	0.0	-	-	+0.3	-0.1	-	+0.1	+0.1

Mean R = 1.0

Mean D = +0.1

Table 41 Invasion and growth of P. falciparum in cells from two Hb E heterozygotes (AE).

CHAPTER 8

DISCUSSION

The haemoglobinopathies and malaria are amongst the most frequently occurring genetic and infectious diseases known to man. For example, up to 1,000,000 deaths per year are said to be due to P. falciparum in Africa alone (Wrld.Hlth.Org. 1975), whilst the majority of homozygous sicklers do not survive to adult life. Moreover, the geographical areas of highest incidence for both these groups of diseases coincide remarkably and, at a cellular level, there is an intimate association between the malarial parasite and the red cell at a time when malaria manifests itself clinically. Hence, it is not difficult to understand how small changes in the constitution of the red cell could have major repercussions on the interaction between red cell and parasite, and therefore determine the final outcome of an attack of falciparum malaria. This in turn might select for those individuals whose red cells offer them some protection from this frequently lethal disease.

This study has sought to define those properties of the red cell which might alter its susceptibility to P. falciparum malaria, and in particular whether the presence of Hb F might play a major role in protecting individuals against mortality from the disease.

PARASITE INVASION OF RED CELLS

The evidence presented in this study showing higher rates of invasion by P. falciparum amongst young red cells, contradicts current literature on this subject. This is to be found mainly in the large textbooks of general and tropical medicine. These almost uniformly maintain that P. falciparum invades red cells indiscriminately as far as the metabolic age of the cell is concerned (Adams and Maegraith, 1960; Conrad, 1971; Bruce-Chwatt, 1971; Maegraith, 1971; Wilcocks and Manson Bahr, 1972; Woodruff, 1974; Esan, 1975; Wintrobe et al, 1975; Edington and Gilles, 1976; Hunter et al, 1976). Remarkably, not one of these authors makes any reference to the original data upon which this statement is made.

Closer examination, particularly of the past literature, revealed that the evidence for indiscriminate invasion by P. falciparum has not at all been clearly established. Results which might have been interpreted as showing a preference by P. falciparum for younger cells, might have been dismissed by comparison with P. vivax. The rate of invasion of reticulocytes by this latter species can be up to a hundred times that of mature red cells (Vryonis, 1939), and the absolute numbers of reticulocytes infected by P. vivax have been found to exceed the numbers of mature cells infected at times during the course of an illness (Kitchen, 1938).

Eaton (1934) was the first to report on red cell age and P. falciparum preference. He examined a single case in which he found a quarter of the parasites present in reticulocytes, which accounted for only 2.5% of the red cells. He even went on to hypothesise that the red cell

was susceptible to the malaria parasite only when it was in the reticulocyte stage - "Anything that increases the actual numbers of reticulocytes favours the progress of the disease." Other workers have also reported a preference by P. falciparum for reticulocytes (Jacobsthal, 1936; Schüffner and de Graaf, 1937; Hegner, 1938). However, data from others have not confirmed this (Baserga, 1937; Malamos, 1937; Matsunobu, 1937; Shushan et al, 1937; Greig, 1938; Kitchen, 1939a and b; Chwatt, 1948).

The two most recent of these reports are of particular interest (Kitchen, 1939a; Chwatt, 1948). Kitchen (1939a) observed the parasitisation of reticulocytes and non-reticulocytes during the course of an infection of falciparum malaria in three cases which had been induced for therapeutic purposes. In two of the three cases, the parasites showed a clear preference for young cells during the early stages of the infection. Nevertheless, of the total of 40 observations made in all three cases, 21 showed a higher infection rate of mature erythrocytes whilst in 18 observations reticulocytes were preferred. Although his data suggested an alternating preference for reticulocytes and then non-reticulocytes, he concluded that P. falciparum was indifferent to the age of the erythrocyte it invaded. Chwatt's study of 1948 has the advantage of dealing with a larger number of cases (45), but only one isolated observation was made in each. Thirty-one of his cases showed non-reticulocytes to be more frequently parasitised than reticulocytes, 13 showed the reverse, whilst in one case there was no difference in parasite distribution. He

concluded that P. falciparum invaded both reticulocytes and more mature cells more or less indiscriminately. However, it should be mentioned that the parasitaemias in the cases he studied were of an extremely low order, and only one of the 45 cases exceeded 1% (1.6%).

All the studies on the age of red cells and its relationship to invasion by P. falciparum highlight the difficulty of interpreting these sorts of results. Most suffer the disadvantage of dealing with only two or three cases in which the parasitaemia is relatively low. In all studies the distinction of young from 'old' cells is made on the basis of one criterion only, i.e. the presence or absence of stainable reticulum. However, reticulocytes represent only a small proportion of the young cell population, whilst non-reticulocytes include many relatively young cells. Thus, distinction of reticulocytes and non-reticulocytes is not synonymous with young and old red cells. Furthermore, the majority of studies do not mention at what stage in the illness blood samples were taken. Reticulocyte response is known to change during the course of an illness (Esan, 1975). Kitchen's data (1939a) certainly showed that the preference by the parasite during the course of an illness fluctuated, so that, whereas reticulocytes were initially preferred in two cases, this preference was later reversed.

Even more important is that these studies do not give an indication as to the parasite maturity at the time counts were done and hence some idea of the length of time the particular parasite had spent in the particular cell after invasion. This is relevant, since the average

reticulocyte in the peripheral blood loses its reticulum after about 24 h (Hillman and Finch, 1967), whilst P. falciparum is present in the peripheral blood for over 36 h of its 48 h cycle, developing from a small to a large ring form (Siddiqui and Schnell, 1972). Thus, a cell containing reticulum when invaded by a parasite might have lost this reticulum and appear as a non-reticulocyte when later counted. In serial samples taken from patients with P. vivax malaria, Vryonis (1939) noted a decreasing parasitisation of reticulocytes with increasing time after invasion had occurred. This he felt could well reflect the loss of reticulum from infected cells, since all the parasites appeared to have arisen from the same invasion process.

These difficulties emphasise the need to examine parasite distribution in reticulocytes and non-reticulocytes as soon as possible after invasion has taken place, when the parasites are still in the small ring stage. They also indicate the need to distinguish red cells on the basis of age by methods more sensitive than the separation into the categories of reticulocytes and non-reticulocytes. Furthermore, examination of cases with higher parasitaemia would increase the accuracy of counts made.

The present study has, as before, used the method of assessing the distribution of parasites amongst reticulocytes and non-reticulocytes in infected blood. However, care was taken to examine the samples at the onset of an acute attack and shortly after invasion of red cells by parasites had occurred. In addition the in vitro culture system

was also employed, which allowed invasion by large numbers of parasites to be studied in an environment free of humoral and cellular immunity, and in particular splenic function, which is known to play a major role in malaria (Coleman et al, 1975). Three methods of separating red cells on the basis of age were used, two of which were more sensitive indicators of cell age than the presence of stainable reticulum inside a red cell. When the distribution of small ring forms of P. falciparum was examined in the blood of patients taken at the onset of an acute attack, a clear preference for reticulocytes was found in all eight cases. Parasitaemias ranging from 1.7 to 13.1% were encountered. Thus, preferential parasitisation of these young red cells was evident, but perhaps not as pronounced as in P. vivax infections.

Infected red cells taken from patients with acute P. falciparum malaria were also separated according to cell age by centrifugation. Centrifugation is now a well established method for separating red cells according to age (Borun et al, 1957; Rigas and Koler, 1961). This was confirmed for the method used in this study by the estimation in the various red cell fractions of enzymes which are known to correlate well with cell age.

If parasites invaded cells indiscriminately, similar percentages of parasites would have been expected in all the red cell age groups (i.e. at the top, middle and bottom of the centrifuged specimen). This would not hold however if parasitisation altered cell buoyancy. In all ten experiments there were significantly more

parasitised cells to be found in the upper layers of the centrifuged specimen. Moreover, no evidence could be found to support alteration of cell buoyancy by the parasite. Multiply-infected cells were evenly distributed in all fractions, and were present even amongst the cells at the bottom as were singly infected cells. The increased buoyancy of reticulocytes on the other hand, was sufficient to result in a virtual absence of these young cells in the bottom fractions.

Nevertheless, a further group of experiments was carried out which eliminated the result being affected by the possibility of alteration of cell buoyancy by parasitisation. Uninfected cells were separated on the basis of age by centrifugation prior to infection in vitro and thus before parasitisation could have any effect on cell buoyancy. Each fraction was subcultured with equal numbers of donor cells. It was found that the number of ring forms emerging in the next generation was significantly higher in recipient cells obtained from the upper fractions compared to those at the bottom. Thus, the merozoites amongst the older red cell population did not achieve as high invasion rates as similar parasites amongst a young population of red cells. This would indicate that merozoites are not capable of invading older cells as an alternative where young cells are not available. This would imply that parasites of P. falciparum do not invade young cells 'in preference' to older cells, but rather that young red cells are more susceptible than older red cells to invasion. This, as will be seen later, has some important implications.

A further means of distinguishing red cells on the basis of age was to use the presence of Hb F inside particular red cells as a marker of cell age. There were several reasons for this. Firstly, this marker can be easily identified visually by the acid elution method of Kleihauer (1957) and has been previously used in studies involving malaria parasites (Diggs et al, 1971). Secondly, Hb F is known to occur in red cells of different ages in several well-defined, naturally-occurring conditions. Thus, in investigations of this kind, which attempted to distinguish the effect of cell age on parasite invasion from the haemoglobin content of a particular red cell, intricate methods of artificially manipulating red cells with regard to their haemoglobin content were obviated. The effects of both Hb F and cell age on the invasion of parasites, and later on parasite growth, could be determined using cells from these conditions. Samples were obtained in which Hb F was contained in cells which were younger (umbilical cord blood cells, $\beta^{o}/\delta\beta^{o}$ thalassaemia), of the same age (British and African forms of HPFH) or older (infants aged 3-4 months) than the cells containing Hb A with which they were compared.

In the blood of young infants with P. falciparum malaria, which were the first Hb F cells to be examined, there were significantly more parasites in the Hb A-containing population both before they had matured to schizonts and after they had matured in vitro, ruptured and reinvaded new cells. On the other hand, when cord or $\beta^{o}/\delta\beta^{o}$ thalassaemic blood cells were mixed with adult erythrocytes in culture, it was clear that the Hb F-containing cells

being younger, were invaded more readily than the Hb A-containing cells in the same culture. Similar results were found when cord cells and adult cells were grown in parallel and not in the same culture well. This once again emphasised that the average age of the red cells present affects parasite multiplication and suggested that in the absence of younger cells, the rates of invasion are reduced.

Cultures containing the cells from the British and African HPFH served as unique controls, since the cells containing Hb F in these conditions have been found to differ from normal Hb A-containing cells only with regard to their haemoglobin content. The average age of the Hb F-containing population in both the British and African HPFH is similar to normal adults. In these cases, no difference could be found in the rates of parasitisation of cells containing either Hb A or Hb F.

It therefore seemed at this stage, that the preliminary finding of a paucity of parasites in the Hb F-containing cells of young infants was due to a preference of the parasite for metabolically young cells. No evidence could be found for failure of infection whereby Hb F might prevent the entry of a parasite into a red cell.

Two other possibilities remained to explain the paucity of parasites seen in the Hb F-containing cells of young infants. One was preferential haemolysis of the Hb F-containing population, particularly those cells with parasites in them (suicidal infection). The other was the early failure of development of parasites once inside

the Hb F-containing cells so that these dead parasites were not visible after staining (abortive infection). The data on preferential haemolysis in this study provide no evidence to support the theory of preferential destruction of the Hb F-containing population. However, the methods used were not sensitive enough to detect minor losses of Hb F-particularly if the losses involved only infected cells and were therefore relatively small. This seems unlikely, since most evidence has shown that all red cells, both parasitised and non-parasitised, are involved in the haemolytic process in falciparum malaria (Esan, 1975). Thus, when it was found in this study that in three cases of falciparum malaria there was no obvious preferential loss of Hb F, it seemed reasonable to conclude that the paucity of parasites found in the Hb F-containing cells could not be accounted for by preferential haemolysis of the cells containing this haemoglobin.

The possible failure of development of a parasite once inside a Hb F-containing cell seems the least likely explanation for the uneven distribution of parasites seen amongst the Hb A and Hb F-containing cells in young infants. Firstly, this distribution was observed for the smallest of parasites very soon after invasion had taken place, and before any significant development had occurred. Secondly, the free merozoite is easily stained, measures approximately 1.5 x 1.0 μ, and can be visualised on light microscopy even before it has invaded the red cell (Aikawa, 1977). Even the parasites which fail to develop once inside the red cell can be seen as pyknotic hyperchromatic forms. Unless

failure of development is followed by almost immediate extrusion in the very earliest stages after reinvasion, the parasites should be visible. Extrusion of a parasite which has failed to develop would in any event be tantamount to a failure of invasion by such a parasite. This could certainly not be a property of Hb F, since cord and $\beta^{o}/\delta\beta^{o}$ thalassaemic cells which both contain almost 100% Hb F, were found to be easily susceptible to parasitisation.

The increased susceptibility of metabolically young cells to invasion by P. falciparum has some important implications in both a theoretical and practical context. Firstly, in any studies which compare parasite counts (i.e. a measure of the density of infection) between either different individuals or the red cell types within the same individual, comparison of the different parasite counts are not valid unless the age and turnover rates of the respective red cell populations are known and taken into account. This may even be important where parasite rates (i.e. the percentage of a population infected) are being compared. This is a relevant observation, because it must throw a certain amount of doubt on many of the published studies which compare parasite rates or counts between cell types, individuals or populations where there is a large variation in the mean red cell age.

Secondly, at a more clinical level the preference of the parasite for young cells, and the resultant diminished multiplication in situations where the average age of cells is older may possibly have an important effect on the course of the disease in patients. The morbidity and mortality

of malaria is directly related to the parasitaemia (Field, 1949). Thus, the relative restriction of falciparum parasites, particularly in heavy infections, to the younger subpopulations of erythrocytes might limit the maximum parasitaemia which can develop and hence the morbidity and mortality of the disease. This preference is usually not as marked as seen in P. vivax infections and in the present study parasites were certainly capable of invading the more mature cells in vitro. This property of the parasite, to invade both mature and immature cells, has often been the explanation offered for the high mortality seen in falciparum malaria infections as opposed to the other species of human malaria which have a predilection for certain red cell populations. This restriction to a certain subpopulation of red cells, limits parasite multiplication and these types of malaria therefore have a low mortality (Miller and Carter, 1976). Certainly in animals infected with malarial species which show a preference for younger cells, there are experimental data which support the hypothesis that average cell age can determine the outcome of infection. Zuckerman (1957) showed that young rats infected with P.vivax had higher parasitaemias and mortality than old rats which have a far lower percentage of reticulocytes. However, when reticulocytosis was induced in the old rats by means of phenylhydrazine, they also developed high parasitaemias with increased mortality. Ladda and Lalli (1966) showed that, in mice, suppression of erythrocytes by blood transfusion significantly decreased the parasitaemia in P. berghei infections.

There are two important situations where the preference of young cells by P. falciparum might be relevant in clinical situations. In the first instance, this may provide yet a further explanation for the observed low incidence of severe malaria in the first six months of life, since a few weeks after birth erythropoiesis virtually stops for two to three months (Bratteby and Garby, 1974). This could reduce parasite multiplication, leading to reduced parasitaemia, and hence lower morbidity and mortality. Secondly, the transient bone marrow depression seen during the most severe falciparum infections in both children and adults (Esan, 1975) might serve to limit parasite proliferation.

A final application of the preference of P. falciparum for young cells lies in the improvement of in vitro culture techniques, where the use of young cells would achieve increase multiplication rates of parasites. This might be useful in circumstances where relatively large quantities of parasite material are required, for example in the production of large quantities of antigen for possible vaccine.

The preference of P. falciparum for young cells that has been demonstrated, raises interesting questions as to the mechanism by which these cells are made more susceptible. Two possibilities seem most likely. Either there are specific sites on the red cell membrane to which the parasite must bind before entering the cell and these are lost as the red cell ages, or the process is an energy-dependent one and relies wholly or in part on the red cell to provide this energy. Since the red cell becomes metabolically effete with age (Borun et al, 1957), an older cell might not be able to

participate fully in the invasion process.

It has long been known that the malarial parasite is highly specific in the type of red cell it is able to penetrate (McGhee, 1953). This might imply the presence of a specific receptor site necessary for invasion (Miller et al, 1973). Beginning at the species level, it is known that a given species of malaria parasite will successfully invade the red cells of only certain animals. Butcher et al (1973) showed that P. knowlesi will only invade the red cells of closely related primate species in vitro which correlated closely with in vivo susceptibility. Red cells of non-primates were resistant even in the presence of sera from primates showing that the restriction of susceptibility was a red cell effect. Miller et al (1973) showed that treatment of human cells with chymotrypsin or pronase could almost eliminate the invasion of P. knowlesi, whereas treatment with neuraminidase had no effect. Treatment of rhesus monkey erythrocytes with these four enzymes had no effect on invasion by P. knowlesi.

Even within a species, there is evidence of differential susceptibility of individuals to malaria which is thought to be due to the presence or absence of so-called receptor sites on the red cell membrane. This has possibly been best exemplified by the presence of the Duffy negative phenotype (one of the blood groups), and the resistance to P. vivax malaria. Miller et al (1975) found that human red cells lacking the antigenic determinants, Duffy a and b (duffy negative) were resistant to invasion by P. knowlesi in vitro. West Africans with the Duffy negative phenotype have been known for some time to be resistant to P. vivax

infections, though susceptible to the other species of human malaria (Bray, 1958). Miller et al (1976) suggested that this phenotype might be the factor involved in resistance. In an experiment involving the exposure of 17 Black volunteers to the bites of P. vivax infected mosquitoes, only those individuals who were Duffy negative were resistant to erythrocyte infection. Even more recently, this group of workers (Mason et al, 1977) have shown that the erythrocytes from three rare non-Negro Duffy negative individuals were resistant to invasion by P. knowlesi in vitro, indicating that the resistance to invasion was mediated by the Duffy-negative phenotype and not another closely linked factor. However, they were unable to show that it was the Duffy antigens themselves that were the erythrocyte receptors for invasion, since a number of cells with no Duffy determinants, either after enzyme treatment or from New World monkeys, were successfully invaded. Thus they concluded that the resistance of human red cells to invasion by P. knowlesi in vitro was mediated by the Duffy-negative phenotype and not necessarily the absence of the Duffy determinants on red cells. Numerous types of human erythrocytes lacking certain blood group determinants have been tested, and failed to block invasion by P. falciparum. This included Duffy-negative red cells (Miller et al, 1977). Whatever the cause, this does highlight the difference in susceptibility amongst individuals to malaria infection.

The difference in susceptibility of young and old red cells in the same individual to invasion by P. falciparum could be explained along similar lines. As with species

and individual specificity, this differential susceptibility could reflect the presence of a receptor site on young red cells which is gradually lost with age. HLA antigens are an example as they are present on reticulocytes but not on mature red cells (Harris and Zervas, 1969; Silvestre et al, 1970). Furthermore, Piazza et al (1972) have shown a remarkable association between the distribution of certain HLA antigens, particularly at the B locus, in areas on Sardinia which were formerly endemic for falciparum malaria. The differences in frequencies of these alleles between the lowland (malarious) and highland villages were significant, although not as much as with thalassaemia and G6PD deficiency as seen in a previous study (Siniscalco et al, 1966).

Alternative to the postulate of a receptor site, Luzzatto et al's evidence (1969) on the preference of young ring forms of P. falciparum for G6PD non-deficient cells might support the argument that the process of invasion is energy-dependant and that this energy must in part come from the red blood cell. Young red cells are certainly known to be metabolically more active than old cells (Borun et al, 1957). Were invasion not an energy dependant phenomenon, it would be hard to imagine how in Luzzatto et al's experiments (1969) a parasite would choose beforehand not to invade a G6PD deficient cell. This is however purely speculative, there being no available data on the energy requirements of parasite invasion.

Finally, there is no evidence to rule out that both a receptor site and energy are prerequisites of parasite invasion, nor for that matter is there any to support it.

These remain rich fields for future work.

PARASITE DEVELOPMENT INSIDE RED CELLS

The initial experiments to assess the relationship between Hb F and parasite growth used red cells from the umbilical cord of healthy newborns. These cells were readily available and hence were the most thoroughly investigated. In most cord blood samples, over 90% of the cells stained for Hb F by the Kleihauer technique. In these cells, a consistent retardation of parasite growth was seen, using three different methods of assessment.

Morphological assessment of cultures containing mixtures of cord and adult cells showed that the more mature forms of the parasite were present in the fully eluted, 'ghost' cells which had presumably contained Hb A. However, this did raise a particularly pertinent question with regard to interpretation, since it was possible that these more mature forms of the parasite might have utilised or eluted all the haemoglobin present, so that a Hb F-containing cell when infected, might appear as a ghost. It has been calculated that for P. knowlesi 75% of the haemoglobin present in a cell is metabolised, all of which could be accounted for by the pigment present in the parasite (Moulder, 1962). This implies that there is no loss of haemoglobin from the infected cells and that it is all metabolised within the cell.

In the present study, morphological assessment of parasites was made when most were in the late trophozoite or immature schizont stage, or even earlier. At these stages, the parasite occupies only a little over half the red cell and thus allows adequate visualisation of the

remaining haemoglobin. However, even mature schizonts were occasionally seen in cells containing Hb F after smears had been prepared by the acid elution technique. Thus, it seemed unlikely that an infected Hb F-containing cell might be classified as an Hb A-containing cell. This problem was finally resolved by using parallel cultures of cord and adult cells. This avoided the need to use the acid elution technique to distinguish the two cell types. All blood smears could be stained by the Giemsa method, which provides the clearest visualisation of detailed parasite morphology. There was still a marked retardation of parasite growth seen in the Hb F-containing cord cells using this method of culture.

The system of parallel parasite culture with different cell types also enabled parasite growth to be measured by the rate of ^{3}H isoleucine incorporation into parasite protein. This amino acid was selected as it has been shown that it is one of the two amino acids, the other being methionine, which the parasite must obtain from outside the red cell (McCormick, 1970; Trigg and Gutteridge, 1971). Furthermore, Hb A contains no isoleucine residues and Hb F has only four such residues per γ chain (Dayhoff, 1972). The uptake of this amino acid was found to be small in the uninfected control cells in all experiments where ^{3}H isoleucine uptake was measured and this was mainly due to contaminating white cells in the recipient blood. Cell for cell, the white cells in cord blood was found to incorporate more isotope than in adult blood. Correction was made for the uptake by uninfected cells and also for the increased parasitisation in cord cells, so that isotope incorporation could be related

to the numbers of parasites present in Hb A-or Hb F-containing cells. This relatively objective and reproducible method of assessment of parasite growth clearly showed that there was more active amino acid incorporation by parasites in Hb A as compared to Hb F-containing cells.

Growth of parasites was also determined by means of weighing the cut-outs of enlarged images of individual parasites in the two cell types. This was done as an additional method to isotope incorporation to measure growth quantitatively. It was clearly shown that after a given time in culture the parasites in the cord cells were markedly smaller than those in the adult cells.

Thus, all the data provided considerable evidence for the retardation of parasite growth in cord cells containing predominantly Hb F. However, even though it appeared the most obvious at the time, the difference in growth of parasites in the two cell types could not be directly attributed to the Hb F itself, because cord and adult cells differ in many other respects than haemoglobin type (Zipursky, 1965; Oski and Komazawa, 1975). Cord cells have a relatively young mean red cell age, their renewal rate is at least three to five times greater than adult cells, they have increased glucose consumption, increased activities of certain enzymes (e.g. most glycolytic enzymes and non-glycolytic enzymes such as glutamic oxalacetate transaminase, glutathione reductase), decreased activities of other enzymes (e.g. phosphofructokinase, carbonic anhydrase, catalase, etc) accelerated decline of 2,3-DPG and ATP when

incubated, increased levels of ATP, increased potassium efflux and many others. Many of these properties appear to be unique characteristics of the newborns erythrocytes, and not merely a function of the presence of young cells. Thus, factors other than Hb F could have accounted for the retardation of parasite growth seen in Hb F-containing cord cells.

It was for the reasons mentioned above that parasite growth was assessed in other types of cells containing Hb F. For example, the $\beta^{o}/\delta\beta^{o}$ thalassaemia double heterozygote provided cells containing predominantly Hb F, which, like cord cells have a rapid turnover and are thus metabolically young. However, these thalassaemic cells are abnormal in many respects which could retard parasite growth (Ottolenghi et al, 1975). Blood from young infants, on the other hand, provides relatively old Hb F-containing cells. Perhaps the unique Hb F-containing recipient cells were those obtained from the British HPFH homozygotes, because no metabolic difference has been found between the Hb F-and Hb A-containing cells of such individuals and thus test and control cells are almost identical except for their haemoglobin content (Weatherall et al, 1975; Boyer et al, 1977).

In all the Hb F-containing cells studied, retardation of parasite growth was seen when compared to Hb A-containing controls. This strongly suggested that Hb F was the factor responsible for the retardation of parasite growth. However, it was still possible that in these conditions the Hb A-and Hb F-containing cell populations might have arisen from different clones of cells, the differences between which have

not been detected. The Hb F-containing populations, for example, might arise from a clone of cells which persist from early infancy (Weatherall et al, 1976). Thus, the conclusion that there is a relative retardation of P. falciparum in Hb F-containing cells of all metabolic ages and regardless of whether they are derived from infants or adults assumes that Hb F-containing cells in infants do not have other metabolic properties which differ from adult red cells; unfortunately, very little information is available on this critical question (Weatherall, 1976). However, the data showing retardation of parasite growth in the African HPFH is helpful in this regard. This condition is the result of a deletion in the γ-δ-β gene complex (Kan et al, 1975) and thus, unlike most other conditions which have raised levels of Hb F, the Hb F is distributed throughout all the red cells (pancellular) in a relatively even manner and is not confined to discrete cell lines. Growth retardation, though not as marked as in cord cells, was seen in these Hb F-containing cells.

The results also provide information as to the amount of Hb F required to bring about retarded parasite growth. Thus, in some of the experiments where the Hb F was heterogeneously distributed amongst the cells, it was possible to define a group of cells which contained intermediate amounts of Hb F as judged by their staining characteristics. The degree of retardation of growth of P. falciparum in these cells lay between that seen in cells containing predominantly Hb F and the controls containing mainly Hb A. In the cells of the African HPFH with about 25% Hb F, growth retardation was detected, but this was not as marked as in other cell types which contained high proportions of Hb F. So, whereas

the mean difference in growth scores (D) between Hb A and Hb F-containing cells in the African HPFH was about -0.5, in the cord cells the average difference in growth scores was of the order of -1.0. These findings suggest that the degree of growth retardation is broadly correlated with the level of Hb F inside the red cell. Since it was not possible to determine accurately the absolute amount of Hb F per cell, the minimal amount of Hb F which is capable of retarding parasite growth could not be determined. However, since the phenomenon was found in the red cells of the African HPFH,where the Hb F is fairly evenly distributed, it seems likely that the effect can result from as little as 7 pg of Hb F per red cell (the average mean corpuscular haemoglobin being 30 pg,and the cells of the African HPFH contain about 25% Hb F).

A further observation was that retardation of growth in Hb F-containing cells was evident at even the earliest stages of the parasites'erythrocytic cycle. This was particularly noticeable in the $\beta^{o}/\delta\beta^{o}$ thalassaemic cells where many ring forms were present. One of the experiments in infant blood, also assessed at an early stage, but then again later, demonstrated how this difference between Hb A- and Hb F-containing cells became more evident with increasing time of development.

It is also of interest that the effect of growth retardation was also seen on the sexual, gametocyte form of the parasite when present in Hb F-containing cells. This difference when judged by weighed cut-outs was even more pronounced than with the asexual forms. This however would

not be entirely unexpected since, assuming that similar processes are involved in the growth of asexual forms, the growth of gametocytes takes much longer than the asexual forms, being about ten days for P. falciparum (Garnham, 1966).

Thus, Hb F has clearly been shown to retard the development of both sexual and asexual forms of P. falciparum in vitro, irrespective of red cell age. This retardation was found to correlate with both the levels of Hb F present and the time spent in culture. Growth restriction was seen in even the most immature of parasites. The overall in vitro evidence presented would therefore support Luzzatto's theory of abortive infection for parasitised cells containing Hb F, since it seems that parasites readily enter such cells, but fail to thrive once inside them.

MECHANISM OF RETARDATION OF PARASITE GROWTH BY FETAL HAEMOGLOBIN

There were several possibilities for the mechanism by which Hb F might cause retardation of parasite growth. The most likely lay in the differences between Hb A and Hb F with regard to oxygen affinity and primary structure.

One of the main functional qualities of Hb F is its high affinity for oxygen. If the parasite were to use oxygen for growth purposes, then in situations where Hb F is present, the parasite might be relatively deprived of this essential requirement. Secondly, Hb F differs considerably from Hb A in primary structure and the parasite might be unable to metabolise it as effectively because of structural differences. Other possible mechanisms,

of growth retardation might be more complex, such as haemoglobin when in the oxy-conformation not being susceptible to parasite degradation. Since Hb F has a higher affinity for oxygen it might therefore be less susceptible to parasite digestion. Such a hypothesis includes both functional and structural aspects of Hb F. Another possibility might be that the relatively high levels of oxygen associated with Hb F might itself be toxic to the parasite. These suggestions remain speculative.

Evidence to confirm the absolute need for oxygen by P. falciparum is crucial to support the hypothesis that the high oxygen affinity of Hb F deprives the parasite of oxygen and in so doing, causes retardation of parasite growth. In a recent review on the subject of oxygen requirements of malarial parasites (Homewood, 1977) the author reaches the conclusion that the intraerythrocytic stages may use oxygen. The main problem has been the failure to successfully free the parasite of contaminating host material such as reticulocytes, white cells and perhaps platelets, all capable of aerobic respiration (Kilby and Silverman, 1969).

The gross overall findings are however similar. Maier and Coggeshall (1941), in in vitro experiments with P. knowlesi, showed an increasing consumption of oxygen by parasitised blood taken at intervals during the maturation from ring to schizont. However, once the parasites were maintained in vitro, their oxygen consumption decreased rapidly. Ball et al (1948), also working with P. knowlesi, confirmed these findings but in addition studied in vivo oxygen consumption. The consumption in vivo was not only far higher than that in vitro,

and increased with the maturity of the parasites, but also increased over the course of a few days' infection with growing parasite numbers. In strong contrast, there was no increase in oxygen consumption by parasites placed in culture. Fulton and Spooner (1956) also found an increased consumption of oxygen by blood infected by P. berghei, and included data on the reduction of oxygen consumption by cyanide and malonate, both inhibitors of oxidative respiration, thus indicating the possible presence of such oxidative pathways in these cultures.

All these studies apply to infected blood as a whole. Thus, increased oxygen utilisation could reflect consumption by components of infected blood other than parasites. Even in blood samples where white cells and platelets are removed, reticulocytes which contain mitochondria, and are therefore capable of aerobic respiration, remain. The in vivo studies have the complication of a fluctuating white cell and reticulocyte population, but they do highlight the shortcomings of the in vitro system with its widely differing results.

Even the studies on some of the component parts of the oxidative pathways in malaria parasites are inconclusive. Thus, attempts to demonstrate a complete functioning tricarboxylic acid (TCA) cycle in malaria parasites have not been successful (Homewood, 1977). Even in avian malaria parasites with cristate mitochondria, this pathway has not been conclusively demonstrated (Aikawa, 1966). Contaminant host material was found to complicate interpretation. In mammalian species of malaria which do not have recognisable mitochondria, the only TCA cycle enzyme detected has been

malate dehydrogenase (Carter and Voller, 1973).

Attempts have also been made to establish the presence or absence of electron transport chain in malarial parasites. Theakston et al (1970) found NADH and NADPH dehydrogenases in P. berghei, but these need not be associated exclusively with the electron transport chain. Ubiquinones are more specific for this pathway and some, not of blood origin, have been found in blood infected with P. lophurae, P. knowlesi and P. falciparum (Schnell et al, 1971). This suggests that these ubiquinones are of parasite origin and might point to the presence of an electron transport chain in these parasites. Scheibel and Pflaum (1970) found evidence for cytochrome oxidase activity in P. falciparum. Other workers have used inhibitors of this pathway to seek evidence of oxidative metabolism in parasites. Thus, inhibitors of the electron transport inhibited the energy-requiring clumping of malarial pigment induced by chloroquine in P. berghei infections (Homewood 1977). Similarly, azide and 2,4-dinitrophenol inhibited the incorporation of ^{32}P-phosphate by free parasites of P. berghei (Nagarajan, 1968).

The present study has used somewhat of a different approach so that instead of measuring oxygen consumption by infected blood, it has looked at the effect on parasite growth of different oxygen tensions or haemoglobins with varying oxygen affinities.

Preliminary experiments attempted to answer what previously seemed a simple question, namely whether parasites had any requirement for oxygen at all. In the course of these studies it was established that under low

oxygen tensions (5% O_2/5% CO_2 in nitrogen) there was in fact an improved invasion rate and growth of P. falciparum which agrees with other workers (Anfinsen et al, 1946; Trigg 1969a); Trager and Jensen, 1976). However, in the absence of oxygen, the results became a little more difficult to interpret.

It was found that parasite growth in supposed anaerobic conditions of 5% CO_2 in nitrogen for 12 h periods was in fact better than under aerobic conditions of 5% CO_2 in air. However, at the same time it was also suspected that the red cells in such 'anaerobic' cultures remained oxygenated for a number of hours as judged by the colour of these cells which were noticeably darker only at the end of such a 12 h period than they were at the commencement. It was therefore considered possible that this period was insufficient for anaerobic conditions to be achieved, particularly amongst the red cells at the bottom of the culture wells. This suspicion was later confirmed when the use of an oxygen monitor showed that cultures exposed to so-called 'anaerobic' conditions required up to 30 h to achieve anaerobic conditions in the culture medium.

When the culture period under anaerobic conditions was extended to 48 h, parasites were unable to grow. They were also unable to clump their pigment in a compact form, a process which is thought to require oxidative metabolism (Homewood, 1977). This might implicate oxygen as a prerequisite for these processes. However there are criticisms of the GasPak anaerobic system used in this study. One is that the level of CO_2 has been shown to fall with time,

because the borohydride residue which is left from the generation of hydrogen in the system is very alkaline and tends to dissolve the CO_2 present (Ferguson et al, 1975). This progressive fall in CO_2 might have affected parasites in the anaerobic jars deleteriously over prolonged periods, both because of pH changes and because parasites are thought to fix CO_2 (Fletcher and Maegraith, 1972). On the other hand, in the cultures opened every 12 h, the generator envelope which evolved the CO_2 and hydrogen was replaced each time. The second criticism of the longer 48 h of undisturbed culture was that if under anaerobic conditions lactate accumulated, for example, then this in itself, or because of pH changes might affect parasite growth unfavourably. Thus, the effect observed might not be directly related to oxygen deprivation. This would presume that the parasite is a facultative anaerobe and produces more lactate under anaerobic conditions than it does aerobically.

One reasonable conclusion from these experiments is that parasite growth in culture is enhanced under conditions of low oxygen tensions. More cautiously it might be said that P. falciparum fails to grow in the absence of small amounts of oxygen and this might demonstrate a requirement by the parasite for oxygen. This agrees with the findings of Trigg (1969a) who cultured P. knowlesi in vitro through one asexual cycle in the absence of, and in 1, 5, 20 and 95% oxygen respectively. Under anaerobic conditions and 95% oxygen, little or no parasite development occurred, whilst there was some enhancement of growth when the parasites were grown in low oxygen tensions (1 and 5%) as compared to

growth in 20% oxygen. More recently, the principle of reducing oxygen tensions for culture work in general has been found to apply to mammalian tissue cultures. Packer and Fuehr (1977) showed that growth of human diploid cells was enhanced at oxygen concentrations less than 20%, and an extension of about 25% on the lifespan of these cells was achieved by long-term cultivation under 10% oxygen. The oxygen tension of 20% (approximately 140 mmHg) most commonly used in cell cultures is unphysiological and certainly higher than that to which cells are usually exposed *in vivo*, arterial oxygen tension in man being about 100 mmHg. These high oxygen tensions might well be toxic.

This has important implications for *in vitro* culture work in general and for improved culture of malaria parasites. Low oxygen tensions have in fact already been incorporated into recently devised methods for continuous culture of *P. falciparum*. At its simplest, a lighted candle is introduced into the culture vessel and using the oxygen monitor, it was shown in this study that this reduces the oxygen tension to about 15%. Haynes *et al* (1976) used 6.6% oxygen, whilst Trager and Jensen (1976) initially used 5%, but later found even better development with 1% oxygen. This study has agreed with the concept that minimal amounts of oxygen are required for the culture of *P. falciparum* and that at such low oxygen tensions growth is in fact better than under aerobic conditions. It might be speculated whether hyperbaric oxygen could be used in very severe falciparum infections.

To test the effects of oxygen deprivation further, a haemoglobin variant (Hb Radcliffe) was used which, as with

Hb F, has a high affinity for oxygen because of a single amino acid substitution in the β chain and not via a mechanism involving 2,3-DPG. Unfortunately, these experiments gave equivocal results. However, in three of the best experiments in this group, no difference was seen in either the pattern of invasion, or growth of parasites in these cells as compared to normal adult controls. It could have been important that these cultures were maintained in aerobic conditions where oxygen was free to diffuse in and out of the system, thus not exposing the cells to any stress with regard to oxygen supply. However, these aerobic conditions were similar to those wherein growth retardation had been seen in cells containing Hb F. Since Hb Radcliffe is functionally rather than structurally similar to Hb F, these experiments did seem to indicate that the high affinity of a haemoglobin for oxygen was an unlikely mechanism for the growth retardation seen in Hb F-containing cells *in vitro*.

The unlikelihood of the high oxygen affinity of Hb F being responsible for retardation of parasite growth was further confirmed when parasites were cultured in Hb F-containing cord cells exposed to low oxygen tensions. It might have been expected that, were the mechanism of oxygen deprivation a real factor, further deprivation of oxygen would enhance any difference in parasite growth between Hb A and Hb F-containing cells. No such effect was observed. Growth retardation under these conditions was of a similar order as seen in aerobic cultures. It seemed therefore that Hb F did not, at least *in vitro*, retard parasite growth because of oxygen deprivation.

The erythrocyte stages of malarial parasites obtain most of the amino acids required for protein synthesis from the digestion of host cell haemoglobin (Fulton and Grant, 1956; Siddiqui and Schnell, 1972). Once the haemoglobin has been taken up into the parasite, it loses those physiological mechanisms which protect it from oxidation and consequently it is auto-oxidised to methaemoglobin which non-enzymically dissociates into haematin and globin (Sherman, 1977). Digestion of the globin portion is carried out by proteolytic enzymes, at least some of which may have been shown to be of parasite origin (Levy et al, 1974). Liberated amino acids can then be incorporated into parasite protein.

It could be possible that Hb F resists degradation somewhere along this pathway and is thus able to retard parasite development. Haemoglobin F liganded to oxygen for example, has been shown to be more stable to alkaline denaturation than Hb A (Painter and Waterman, 1973) and this points to one difference in the degradation of these two haemoglobins which could be relevant to malaria parasites. Very little is known of how the parasite deals with haemoglobin, particularly the proteolysis of the globin moiety by parasite enzymes. This seems to be a promising area for further study in order to determine the mechanism by which not only Hb F, but also other haemoglobin variants might protect the red cell in P. falciparum infections.

Other possible mechanisms remain extremely speculative. The first is that of the inability of the parasite to degrade

haemoglobin when in the oxy-conformation. As explained in the introduction, the first step in the digestion of haemoglobin by the parasite is the dissociation of this molecule into its two major components, haem and globin (Moulder, 1962) and if this can be affected by the bound oxygen resulting in a structure which cannot be degraded by the parasite, then it could provide a mechanism which involves both functional and structural aspects of Hb F in retarding parasite growth. Such a hypothesis has no experimental basis.

A second suggestion, that high levels of oxygen are toxic to parasites and hence Hb F, because of its high affinity, retards parasite growth, is possibly a little better founded. The data presented have shown that parasites of P. falciparum grow better, at least in vitro, under conditions of lowered oxygen tension. Furthermore, concentrations of 95% oxygen have been found to be toxic to parasites (Trigg, 1969a). It is now known that the presence of oxygen and its binding to haemoglobin can retard the rate of glycolysis in red cells (Asakura et al, 1966) and it is particularly in red cells containing parasites that glucose transport and metabolism is increased (Homewood and Neame, 1974). All these factors could support a mechanism whereby Hb F with a high oxygen affinity inhibits glycolysis necessary for the highly metabolically active malarial parasites. This is not supported by experiments involving Hb Radcliffe. Data in this study and others (Maier and Coggeshall, 1941; Ball et al, 1948) have shown that the metabolic activity of parasites increases dramatically with maturity and is most marked in P. falciparum infections

with a short erythrocyte cycle. It might be for this reason that P. falciparum particularly should seek out the deep tissues where the oxygen tension is low in order to allow maximal glycolysis for its energy requirements. This would not, however, explain the in vitro findings of this study where both Hb A and Hb F would be fully saturated with oxygen under the aerobic culture conditions.

The data presented have shown retardation rather than inhibition of parasite growth in cells containing Hb F. Mature schizonts were occasionally seen in these cells. How could this relative retardation of parasite growth therefore protect in malaria? Firstly, it must be remembered that clinical malaria only becomes evident after a number of erythrocytic cycles of the parasite which follow the release of merozoites from liver schizonts. During the first few cycles in the blood the parasite may not even be evident on thick blood film. Thereafter a period follows when the parasite is present if the blood is examined microscopically but the patient remains asymptomatic. Thus, in this prepatent period before the patient becomes ill and later during the illness before any complications of the disease have arisen, the parasite has spent a considerable time in the blood, during which it has gone through numerous cycles of multiplication in the red cells. This occurs particularly in individuals who have built up some acquired immunity (Maegraith, 1971). Cumulatively, small effects in terms of growth retardation might be significant in allowing for example, humoral and cellular defences of the host to be mounted against the parasite.

The consistent finding of more abnormal and degenerate forms of parasites in cells containing Hb F possibly provides a more rational mechanism for the attenuation of malarial infections, as this would obviously decrease overall parasite multiplication. Since the serious complications of the disease are related to the density of parasites in the blood (Field, 1949), this effect of reduction of multiplication would most certainly be protective. More direct evidence of the decreased multiplication of parasites in Hb F-containing cells was obtained when the parasites arising from an erythrocytic cycle in Hb F-containing cord cells were compared to those in Hb A-containing cells, and found to be significantly fewer in number. This occurred despite the increased number of parasites present in the cord cells during most of the culture period. However, the results of this experiment might reflect one of the shortcomings of this short-term culture system in that it usually shows little, if any, increase in parasite numbers in the third generation *in vitro* (Phillips *et al*, 1972). Furthermore the increased numbers of parasites seen in the cord cells in the second generation might have depleted the culture medium of essential nutrients in those wells with cord cells and thus lead to a large reduction in parasite numbers in the next generation *in vitro*.

The results of the present study also clearly show that where there is a mixed population of Hb A-and Hb F-containing cells, there is a pronounced loss of synchronicity of parasite maturation in excess of that seen in the *in vitro* culture system itself, because of the retardation of growth

in the Hb F-containing cells. This could be important in the evolution of cerebral malaria, one of the main causes of death in P. falciparum infections, since highly synchronous rupture of schizonts might cause more cerebral damage than situations in which this process is staggered. A loss of synchrony in parasite rupture as caused by Hb F might protect by reducing the severity of cerebral malaria.

Finally, it cannot be discounted that these differences observed in vitro might be even more exaggerated in vivo and thus provide a clear mechanism of protection. For example, the oxygen consumption of parasites in vivo has been found to be far higher than that in vitro (Ball et al, 1948) and many other differences may exist in which the in vitro situation does not reflect the situation in the infected patient. Fetal haemoglobin, via more obvious mechanisms, may be life-saving in such situations.

Whatever the mechanism by which Hb F retards growth of P. falciparum in vitro, confirmation of a similar effect in vivo would have far reaching implications. Firstly, it could provide a further means by which newborns living in a malarious area might be protected against fulminating infections during the first six months of life when the incidence and mortality from malaria is low (Bruce Chwatt, 1952). Protection of newborns is already believed to include passive immunity acquired from the mother (Logie et al, 1973 para amino benzoic acid deficiency due to a milk diet (Ferone, 1977) and selective biting by mosquitoes (Muirhead-Thomson, 1951). Further suggestions have been an immature liver unable to support the pre-erythrocytic cycle of the parasite

and the custom of keeping babies covered by clothing. These are well reviewed by Colbourne (1959). It does therefore seem fascinating that Hb F should have a role to play in the presence of so many other potential means of protection. The strongest contender would however seem to be passively acquired immunity from the mother, but even with this means of host defence, a number of inconsistencies exist.

Pregnancy has now been clearly established as a period of increased susceptibility to falciparum malaria (Bruce Chwatt, 1952; Gilles et al, 1969). Bruce Chwatt (1952) recorded a mean parasite rate of 33% primarily due to P. falciparum amongst partur ient women in Ibadan which was 12% higher than in the general population. Parasite counts during pregnancy were found to be ten times higher than in the same individuals before conception or in a non-pregnant control group (Gilles et al, 1969). This might suggest that newborns would be equally if not more susceptible to malaria than their mothers. And yet congenital malaria is almost unheard of (Logie et al, 1973) and remains rare for the first six months of life (Garnham, 1949). This might suggest that passively transferred immunity is not the only factor involved in protection against malaria in infancy. McGregor et al (1965) have shown that in infants by about the fifth week after birth, antibodies with anti-parasitic activity, as measured by fluorescent antibody techniques have reached exceedingly low levels. This is far faster than is generally accepted for the loss of resistance to infection. There is very little evidence in fact which demonstrates the protective effect of passively transferred

immunity from mother to child. In vivo, the only possible attempt to confirm that the high levels of gamma globulins protected was by Edozien et al (1962) who showed that the purified globulin obtained from the umbilical cord of a newborn, born of a mother immune to falciparum malaria, was capable of suppressing a falciparum infection in a 12-month-old child. However, this cord gamma globulin took much longer to act than that prepared from immune adults. These sorts of data serve mainly as pointers to the inadequacy of passively transferred immunity as a full explanation for the low incidence of malaria seen during the first few months of life.

Data presented in this thesis showed that sera taken from the umbilical cords of newborns of mothers living in an area endemic for P. falciparum malaria inhibited parasite invasion of red cells to some extent in vitro compared to sera from newborns of non-immune mothers. However, in some of the so-called immune sera no inhibition of invasion was seen, whilst in others there was enhanced invasion. These results would nevertheless indicate that, at birth at least, the sera from newborns in areas endemic for malaria have some anti-parasitic activity in their serum. However, it is critical to establish for what length of time after birth this protection is sustained.

INVASION AND DEVELOPMENT OF PARASITES IN OTHER RED CELL DISORDERS

This study has been concerned mainly with the interaction of P. falciparum and the red cell containing Hb F. However, much of the convincing data presented in the introduction to

this thesis have dealt with the evidence for protection against malaria seen in each of the common haemoglobinopathies. These data have been mainly of an epidemiological nature and the actual mechanism involved in protection has remained speculative. Consequently, it seemed only reasonable to investigate these red cell disorders in a similar manner to the methods employed for Hb F, using the in vitro culture system that has been described here.

Of the common inherited disorders, epidemiological data presented to support the hypothesis of protection of thalassaemic heterozygotes against P. falciparum malaria are the least convincing and the least documented. This is particularly surprising as it was the condition first implicated, at the theoretical level at least, in the malaria hypothesis (Haldane, 1949). It was for these reasons that culture of parasites in these cells was carried out to see if they had any deleterious effect on the parasite at all. Because of their small size and low haemoglobin content it was felt they might affect parasite growth deleteriously. Cells from patients with iron deficiency were included because they resembled the thalassaemic cells, at least inasmuch as their size and haemoglobinisation was concerned. Data on the cellular interaction of parasite and cells found in these two conditions would also clarify the relative importance of the raised levels of Hb F seen in early life in thalassaemia (Beaven et al, 1962; Alter, 1978; Serjeant, personal communication). Any detrimental effect of thalassaemia trait cells observed on parasites would tend to shift the emphasis of protection in this condition from the Hb F present in infancy to the properties of these abnormal

cells.

At the outset it did seem likely that the small under-haemoglobinised cells seen in β thalassaemia trait and iron deficiency might be unfavourable to the parasite. If P. falciparum for example were to metabolise 75% of the haemoglobin in each cell, as with P. knowlesi (Moulder, 1962), then the intracellular haemoglobin in a thalassaemia trait cell, having only about 66% of the haemoglobin of a normal adult cell (Weatherall and Clegg, 1972), might be inadequate to supply the parasites' needs. However, it is known that amino acids are transported into the malaria-infected cell (Sherman, 1977a) and thus host cell haemoglobin might not exert any growth-limiting effect in the presence of an adequate extracellular source of amino acids. An alternative basis for retardation of parasite growth might be that the small cells in thalassaemia and iron deficiency anaemia could place physical limitations on parasite size. These are only two of the many other aspects in which cells in β thalassaemia trait differ from those in normal adults and which could account for growth differences.

It was therefore surprising when no evidence for decreased invasion or retarded development of P. falciparum was found in both cells from carriers for thalassaemia and in iron deficient patients. It should be noted that the medium contained both an adequate supply of amino acids and iron, so that it was the aspects of haemoglobinisation and cell size that were being examined rather than the supply of extracellular amino acids or free iron. The rates of invasion in the thalassaemic cells were in fact minimally,

but consistently raised compared to the controls. This is in keeping with the parasite's preference for young cells, as the ^{51}Chromium half life in thalassaemia trait has been shown to be 23 days, slightly below the normal range of 25-35 days (Pearson et al, 1960; Malamos et al, 1961). The main criticism of the experiments with both of these cell types is that a full cycle of parasitic development could not be observed in them due to the short term nature of the cultures. This is important, because the deleterious effect of cell size or inadequate amounts of haemoglobin may only occur with the most mature parasites, just prior to schizont rupture. However, at the same time it should be added that in some of the cultures mature schizonts were observed in these small cells and appeared quite normal.

The data on iron deficiency and malaria are scanty. In a study on the various infections observed in anaemic patients, Masawe et al (1974) found that 24% of those individuals with a negative marrow stain for iron had malaria, whereas this figure was only 5% for those with iron positive stains. This however gives no indication as to the relative susceptibility of iron deficient patients to malaria as there was no non-anaemic control group. They did observe that with both oral and intramuscular iron replacement, the parasite count in those iron deficient patients with malaria increased. However, no details of the iron dosage or parasite counts are given.

As further confirmation of increasing parasitaemia with increasing serum iron, another study (Murray et al, 1975)

noted that patients admitted to hospital for reasons other than malaria commonly developed attacks of malaria. They attributed this to the change in diet in the hospital environment. In their study there was an early rise in serum iron and transferrin saturation on day 2, followed by a peak in parasites on day 5. However, they also noted that the reticulocyte count rose dramatically and therefore this increase in the younger cell population might well explain the increase in parasite multiplication.

In both of these studies, the increase in dietary or serum iron may have been incidental, whilst the presence of young cells might offer an alternative explanation for the flaring up of the malaria. The latter study (Murray et al, 1975) did attempt to confirm their findings by using an animal model. They found that 20 Wistar rats given a dose of 10 mg intramuscular iron dextran two weeks prior to infection with P. berghei had a more rapidly rising parasitaemia and died earlier than a similar group which had received normal saline. Unfortunately, no indication is given as to the iron status of the rats before the experiment or of the mean red cell age in each group after iron was administered. They believed that the explanation of their findings was the requirement of the parasite for free iron.

These data remain inadequate to establish whether iron deficiency is a state of increased or decreased susceptibility to P. falciparum infections. Certainly none of these studies have looked at iron deficient individuals and compared them with a non-deficient normal group. Nor have iron deficient cells been looked at from

the point of view of their structural attributes and interaction with the parasite. This study has concentrated on these latter aspects of the red cells from iron deficient patients inasmuch as they resemble cells from individuals with thalassaemia trait. No reduction of parasite invasion or retardation of parasite growth was seen in either of these types of hypochromic microcytic cells. If these *in vitro* observations reflect the *in vivo* situation in any way, then they have found no mechanism based on properties of thalassaemic trait cells alone which could account for protection of heterozygotes for this condition against falciparum malaria. As such, they re-emphasise the possible importance of the persisting levels of Hb F in this sort of protective role.

The experiments carried out in this study with cells from the sickling disorders demonstrate that while there is minimal retardation of invasion and growth of *P. falciparum* in cells containing Hb S maintained under aerobic conditions, a marked reduction in both invasion and development occurs when these cells are exposed to conditions of low oxygen tension. It seems very likely that this effect is due to the properties of Hb S because exposure of normal erythrocytes to reduced oxygen tension if anything potentiated parasite invasion and growth. Furthermore, it is evident that to elicit the effects of reduced oxygen tension on the rates of invasion and growth of *P. falciparum* in cells containing Hb S the cells need not sickle; a low oxygen tension is sufficient.

There is good evidence that cells containing Hb S undergo significant physical changes under reduced oxygen tension before sickling is demonstrable (Messer et al, 1975; Hahn *et al*, 1976).

Areas of electron dense material become visible on electron microscopy and the cells become more rigid as shown by their inability to pass through 3 μ pores in polycarbonate sieves. It seems likely that some of these physical alterations on exposure to low oxygen tensions could be responsible for the reduced rate of invasion and growth of P. falciparum in Hb S-containing cells.

These observations suggest a mechanism for the protection of sickle cell heterozygotes from P. falciparum malaria in vivo. After development of schizonts inside the liver, the liberated merozoites invade red cells. The resultant ring forms mature quite readily in well oxygenated peripheral blood where they normally spend the first 36 hours of the erythrocytic cycle (Siddiqui and Schnell, 1972). After this the infected cells retreat to the deep tissues where the parasites mature to schizonts during the last 12 hours of the cycle. During this period, the cells are exposed to oxygen tensions very similar to those which cause retardation of parasite development in the present experiments. Hence in red cells containing Hb S this stage of parasite maturation might be retarded. Furthermore the inhibition of invasion of released merozoites into Hb S-containing cells under these low oxygen tensions would reduce the degree of parasite multiplication. If the parasitaemia were to increase, the resulting inflammation and blockage of the capillaries in the deep tissues would increase the degree of vascular stasis and anoxia (Maegraith and Fletcher, 1972) and hence provide an even more unfavourable environment for parasites in cells containing Hb S.

This proposed mechanism for the protection of sickle cell heterozygotes against P. falciparum has several attractions. It explains why protection is predominantly against P. falciparum malaria since this is the only malaria occurring in humans in which the parasite is confined for at least part of its erythrocytic cycle to deep tissues where the oxygen tension is low. It also explains why individuals with sickle cell trait remain susceptible to infection by P. falciparum but how increasing parasitaemia works to the detriment of parasite survival. This would be in agreement with the many epidemiological studies which have shown that sicklers can become infected but that high parasitaemia and subsequent mortality is rarer amongst such individuals (Raper, 1956; The Lambotte-Legrands, 1958). Furthermore, the mechanism does not rely on the sickling of AS cells to produce a detrimental effect on the parasites. This is important because it has always seemed unlikely that in vivo sickling occurs to any marked degree at physiological oxygen tensions in sickle-cell heterozygotes (Milner, 1974).

Unlike previous investigations, a deleterious effect of Hb S on parasites has been clearly demonstrated in vitro. In the experiments of Luzzatto et al (1970), parasitised AS cells were shown to sickle more rapidly than unparasitised control AS cells in 8% sodium metabisulphite, and they presumed that this occurred because the parasites had utilised oxygen from the red cells. They suggested that this phenomenon might occur in vivo and that the parasitised sickled cells might be preferentially removed from the circulation and hence the parasite could not complete its life cycle. However, it seems unlikely that a parasite can cause sufficient

reduction in oxygen tension in an AS cell to cause it to sickle and remain sickled in peripheral blood. A mechanism whereby the parasite's capability for invasion or growth in AS cells in areas of reduced oxygen tension is limited, and where this effect can occur without these cells necessarily sickling, seems a more likely basis for the protection.

Friedman (1978) has recently shown that malarial parasites maintained in continuous culture do not develop in AS or SS cells under conditions of reduced oxygen tension. However, he found that this effect was only demonstrable with young parasites and not with mature forms. This fails to explain why Hb S should protect against malaria, since these immature forms are found mainly in well oxygenated peripheral blood. In the present experiments, it was found that growth retardation occurred in the more mature forms as well. It is difficult to understand the discrepancy between the findings of this study and those of Friedman, but this study has indicated that, in stationary cultures at least, the oxygen tension in the medium may take up to 30 hours to equilibrate with the surrounding gaseous environment. Friedman's experiments were conducted in stationary petri dishes and this might explain his failure to observe retardation of growth of mature parasites in cells containing Hb S. This prolonged period of equilibration of oxygen tensions between gas and medium might also explain why in some cases in the present study invasion of AS cells under low oxygen tensions was not markedly reduced, since the process of red cell invasion took place shortly after the commencement of the subcultures.

Despite these shortcomings, these experiments have demonstrated that at the cellular level Hb S has a profoundly detrimental effect on the proliferation and, in particular, on the growth of P. falciparum when exposed to low oxygen tensions.

The few preliminary results in the present study with cells from individuals deficient in G6PD showed no difference in the invasion of parasites in vitro into cells deficient in G6PD activity when compared to normal adult cells. However, the development of parasites once inside them was significantly retarded. Two precautions must be taken into account. Firstly, the effects of incubation at 37°C on G6PD activity in both normal and deficient cells have yet to be defined and, secondly, there were a large number of abnormal parasites seen in even the control cells in this particular group of experiments.

Nevertheless, these results do concur with other studies carried out at the cellular level with G6PD deficient cells, in as much as they confirm a deleterious effect of a deficiency of this enzyme on malarial parasites (Luzzatto et al, 1969, Eckman et al, 1976; Eaton et al, 1976). What is not altogether clear is the mechanism involved. Thus, Luzzatto's data on the preference of P. falciparum for non-deficient cells in infected patients favour any three of his proposed mechanisms of failed, abortive or suicidal infection since they only look at the distribution of parasites amongst the cell types. Eckman and Eaton's work on the other hand on vitamin E deficient mice infected with P. berghei, favours a mechanism of suicidal infection whereby oxidant induced haemolysis of infected cells occurs before the parasites can mature fully. The present study may therefore seem to

complicate the picture, since it was found that parasites did not grow optimally in the enzyme deficient cells, thus implicating the mechanism of abortive infection. However, it should be added that these experiments have the limitation that the mechanisms for the removal of particular cells are not available *in vitro*. The spleen, for example, plays a particularly important role in removing parasites from infected cells (Conrad and Dennis, 1968; Conrad, 1969). Thus, there is the possibility that the *in vivo* counterpart to failure of development in culture would be the removal of cells containing abnormal parasites by the reticuloendothelial system. It is even possible that parasitised enzyme deficient cells in Luzzatto's experiments were prematurely removed, thus accounting for the paucity of parasites in the deficient population. Thus, the overall data for G6PD deficiency and *P. falciparum* might be compatible with a mechanism of suicidal infection of parasitised cells for the protection of deficient individuals against malaria. Unfortunately, each type of evidence, *in vivo* in humans (Luzzatto et al, 1969) in an animal model in rats (Eckman *et al*, 1976; Eaton *et al*, 1976) and *in vitro*, as in this study, has its own inherent weaknesses and thus the cellular data still remain inconclusive at present.

The process of invasion is still not sufficiently well understood to determine whether G6PD is required for this process, although this enzyme is important in red cell membrane integrity (Lehmann and Huntsman, 1974). Growth retardation of parasites might occur if the enzyme or its products were essential for nutrition. This is especially important since it has been shown that

P. falciparum does not possess any G6PD activity itself and if required is dependent on the enzyme of the host cell (Theakston et al, 1976). These authors, using electron microscopic techniques, found evidence for the presence of this enzyme in the host red cell and in the food vacuole of the parasite but not in the parasite cytoplasm itself. Even if the parasite were not dependent on G6PD activity, then it might indirectly require the products of the hexose monophosphate pathway of which G6PD is a key enzyme. Reduced glutathione is just such a product which has been suggested as the key factor in the protection seen in G6PD deficiency (Motulsky and Campbell-Kraut, 1961) as it has been found essential for parasite growth (McGhee and Trager, 1950). Other products, such as pentose sugars required for nucleic acid synthesis could also be important. Thus, there are many mechanisms by which a G6PD deficient cell could be inhospitable to a parasite and protect the host against malaria. The data are, of course, at present still incomplete.

FINAL CONCLUSIONS

The findings described above have demonstrated possible cellular mechanisms for the protection of individuals with the sickling disorders and G6PD deficienty against fatal malaria. However, no evidence for the mechanism of protection was observed in some of the other very common β chain haemoglobinopathies, such as Hbs C and E, and thalassaemia. This sort of evidence tends to emphasise the possible importance of Hb F in these latter conditions since this haemoglobin has been shown on the present study to retard parasite growth. Critical to this

argument at present is the need for more confirmatory data on the delayed rate of decline of Hb F in infants heterozygous for these conditions. Such crucial data seem to be now forthcoming from studies conducted in Jamaica (Serjeant, personal communication). Similar studies are underway in London (Weatherall, personal communication) and, if they confirm the initial reports, will lend weight to the relative importance of Hb F in the reduction of mortality from malaria in carriers of these haemoglobinopathies. This does, of course, depend on the *in vitro* observation of the effects of Hb F being demonstrated *in vivo*.

This hypothesis invoking Hb F in the protection against malaria has many attractions. Haemoglobin F is present at an age when mortality from malaria is at its highest and acquired immunity is at its lowest. Evidence for protection by Hb F against malaria would also partly explain the still puzzling question of why the incidence of malaria is so low even in normal infants during the first few months of life. Whilst the hypothesis does not exclude other protective mechanisms that have been suggested for other haemoglobinopathies, it does provide a unifying mechanism of defence for many of the different heterozygotes in this broad spectrum of disorders. Consequently, it offers a feasible explanation for the unexpectedly high gene frèquencies observed in many of the inherited red cell disorders and in particular β thalassaemia, one of the commonest of all.

PROPOSALS FOR FUTURE WORK

Over the next few years it is hoped to continue and expand upon the work outlined in this study dealing with P. falciparum and the red cell.

1. It would be of interest to know which properties of Hb F are responsible for the retardation of parasite growth. Two possibilities have been emphasised in this study, viz. relative oxygen deprivation by this haemoglobin with a relatively high oxygen affinity, or a relative inability by the parasite to metabolise Hb F.

The first of these possibilities requires more basic data on the oxygen requirements of the parasite both in vitro and in vivo. The in vitro situation requires more careful monitoring of the oxygen tension present in the cultures and the oxygen consumption by the parasite. Could the parasite for example be a facultative anaerobe? The second possibility is probably far more complex and involves a study of how parasites degrade haemoglobin, particularly with regard to proteolytic enzyme digestion of the globin fraction.

2. The recent methods for continuous culture of malarial parasites would make it possible to observe the cumulative effects on the multiplication of parasites over a number of cycles of growth, not only in Hb F-containing cells but in other red cell disorders.

3. It would also be important to determine what level of Hb F within an individual red cell is required to retard parasite growth, since studies of the heterozygotes for

the haemoglobinopathies have to date shown only a moderately raised level of Hb F during infancy. This will require analysis of red cells obtained from adults with a variety of genetic disorders where the intracellular level of Hb F is known or can be determined.

4) Confirmatory evidence for the retardation of the decline of Hb F in infants heterozygous for β thalassaemia or structural haemoglobin variants is critical if the hypothesis outlined here, regarding the maintenance of certain high gene frequencies, is to be strengthened. This work is currently being undertaken in collaboration with groups in Jamaica and London and seems promising.

5. There are at least two likely mechanisms to explain the preferential invasion of young cells by P. falciparum which have been described in this study. The first involves the search for a possible receptor on the red cell membrane which facilitates the invasion by P. falciparum. The pre-treatment of red cells with various enzymes before placing them in culture seems a reasonable initial approach. Secondly, the process of invasion may be energy-dependant, so that subtle differences in a parameter such as ATP regeneration may be critical for the parasite to penetrate the red cell. Possibly the most direct approach to this problem would be to artificially deplete the red cell of, say, ATP and examine the relative rates of parasite invasion in vitro.

6. The data presented on G6PD deficient cells and parasite growth and invasion are not entirely satisfactory. It might also be worthwhile to examine parasites in cells from iron deficient patients where the medium for culture is also free

of iron. Ovalocytosis is also common in parts of the world where falciparum malaria is endemic and these cells could be tested using this system of short-term parasite culture.

7. This thesis has also highlighted the inadequacy of the knowledge of the anaemia seen in falciparum malaria. A study which will hopefully present the total haematological picture rather than a few isolated parameters is shortly to be undertaken by the Nuffield Department of Medicine.

APPENDIX A

MATERIALS USED

Chemicals

All chemicals for general use, salts, solvents, acids etc were Analar grade unless specifically stated otherwise and were obtained from B.D.H. Chemicals Ltd.

Parasite culture

Tissue culture medium 199 (modified) with Earle's salts was a product of Flow Laboratories Ltd. and was supplied in powder form.

Ingredient	mg/litre	Ingredient	mg/litre
L-alanine	25.000	ATP,NA_2	10.000
L-arginine.HCL	70.000	5-adenylic acid	0.200
L-aspartic acid	30.000	L-ascorbic acid	0.050
L-cysteine.Mcl.H_2O	0.110	D-biotin	0.010
L-cystine	20.000	Calciferol	0.100
L-glutamic acid	66.820	D-Ca pantothenate	0.010
L-glutamine	100.000	Choline Chloride	0.500
Glutathione	0.050	Folic acid	0.010
Glycine	50.000	i-inositol	0.050
L-histidine.HCl.H_2O	21.881	Menadione	0.010
Hydroxy-L-proline	10.000	Niacin	0.025
L-isoleucine	20.000	Nicotinamide	0.025
L-leucine	60.000	p-aminobenzoic acid	0.050
L-lysine.HCl	70.000	Pyridoxal.HCl	0.025
L-methionine	15.000	Pyridoxine.HCl	0.025
L-phenylalanine	25.000	Riboflavin	0.010
L-proline	40.000	Thiamin.HCl	0.010
L-serine	25.000	DL-X-tocopherol-phosphate Na_2	0.010
L-threonine	30.000	Vitamin A acetate	0.1147
L-tryptophan	10.000	Cholesterol	0.200
L-tyrosine	40.000	2-Deoxy-D-Ribose	0.500
L-valine	25.000	D-ribose	0.500

Ingredient	mg/litre
Adenine sulphate	10.000
Guanine.HCl	0.300
Hypoxanthine	0.300
Thymine	0.300
Uracil	0.300
Xanthine	0.300

Ingredient	mg/litre
Na Acetate $3H_2O$	60.000
Tween 80	5.000
$Fe(NO_3)_3.9H_2O$	0.100
Earle's Salts	
$CaCl_2$	200.0
Dextrose D-Glucose	1000.00
$MgSO_4$	98.0
KH_2PO_4	-
NaCl	6800.00
$NaH_2PO_4.H_2O$	140.00
Na_2HPO_4	-
KCl	400.00
Phenol Red Na	17.00

The supplemented medium was made up as follows:

Tissue culture medium 199	10 gms
Glucose	2 gms
Sodium bicarbonate	2.2 gms
Streptomycin	(100,000 µg/l)
Penicillin	(200,000 units/l).

This was made up to a litre with distilled water. It was sterilised by filtration through a 0.22 mu Millipore filter, bottled in 95 ml aliquots and stored for up to a month at 4°C. Before use 5% v/v fetal calf serum was added.

Fetal calf serum (FCS) was supplied by Flow Laboratories Ltd. and was stored at -20°C. It was not heat inactivated before use.

AB serum (non-immune) was obtained from the Oxford Regional Blood Transfusion Service and was stored at -20°C. It was not heat inactivated before use.

Hepes buffer (N-2-hydroxyethyl-piperazine-N'-2-ethanesulphonic acid) was obtained from Flow Laboratories Ltd. and stored at $4^{o}C$.

Whatmans CF 11 cellulose powder was used to remove white cells.

Flat-bottomed plastic microtissue culture plates were obtained from Linbro Chemical Co.Inc.

Scintillation counting

Radioactive isoleucine L-4,4(n)-^{3}H Isoleucine (specific activity 17 Ci/mmole (M Wt 131.2) aqueous solution in 2% ethanol was obtained from the Radiochemical Centre, Amersham.

Hydrogen peroxide 30% w/v

Tissue solubiliser NCS ^{TM} A solution of quarternary ammonium base in toluene was obtained from Amersham/Searle, High Wycombe.

PPO (2,5-diphenyloxazolyl) and POPOP (1,4-bis 2-(5 phenylozazolyl) Benzene) was obtained from Packard Instrument Co. Inc.

Scintillation fluid was prepared as follows: PPO - 4 gms and POPOP 10 mg per litre of scintillation grade toluene.

Staining

All stains were obtained from G.R. Gurr Ltd.

Giemsa stain was obtained in concentrated liquid form. For normal use it was diluted to a 5 or 20% solution with a buffer (pH 7.2 - 7.4) suitable for malaria parasites. The buffer contained $Na_2 HPO_4$ 3 gms/l and $KH_2 PO_4$ o.6 gms/l of

distilled water.

Eosin obtained in powdered form was used as a 1% w/v solution in distilled water.

Stevenels Blue stain was prepared from methylene blue and potassium permanganate. It was made up as follows:

(A)	Methylene blue	1 gm
	Water	75 ml
(B)	Potassium permanaganate	1.5 gm
	Water	75 ml

The two solutions (A and B) were mixed in a flask and placed into a boiling water bath until the precipitate had dissolved (this is important and could take up to two hours). The mixture was allowed to stand until at room temperature and filtered.

Brilliant Cresyl blue 1 gm in 100 ml citrate saline (one part sodium citrate 30 gms/l to four parts normal saline).

Elution buffer for Kleihauer technique (Kleihauer et al, 1957)

$Na_2 HPO_4$	7.55 gms
Citric acid	15.43 gms

These were dissolved in a litre of distilled water. The pH of the buffer was adjusted with IN NaOH. A pH of 3.35 is preferable where varying amounts of Hb F are contained within the cells (mixtures of cord and adult cells). The buffer was stored at $4^{o}C$ for up to a month.

Phosphate buffered saline (PBS)

$Na_2 HPO_4.12H_2O$	60 gms
$Na\ H_2PO_4.2H_2O$	13.6 gms

These were dissolved in a litre of normal saline and stored at $4^{o}C$. When required 40 ml of this stock solution was made up to one litre with normal saline. The final pH was 7.3.

Plasmagel (3% gelatin in normal saline) was made by Laboratoire Roger Bellon, 159 Avenue du Roule, Neuilly (Hauts-de-Seine) France and supplied by Micro Bio Laboratories Ltd., 46 Pembridge Road, London NW11. It consists of:

Modified gelatin	3g
NaCl	0.7g
$CaCl_2.2H_2O$	0.2g
Distilled water	100 ml.

APPENDIX B

BLOODS USED

Blood		Obtained from:	Acknowledgements to:
Malarious		MRC Outpatients, Fajara, The Gambia	Mr. A.K. Rahman
Normal adult	(a)	MRC Outpatients, Fajara, The Gambia	MRC Staff
	(b)	Radcliffe Infirmary, Oxford, U.K.	Prof. D.J. Weatherall
Cord	(a)	Royal Victoria Hospital, Banjul, The Gambia	Sister P. Shingles
	(b)	The German Sisters Mission, Pipeline Rd., Serrakunda, The Gambia	Dr. H. Ropp
	(c)	The John Radcliffe Hospital, Oxford, U.K.	Sister R. Quick
$\beta^{o}/\delta\beta^{o}$ thalassaemia		Stoke Mandeville Hospital, Aylesbury, U.K.	Dr. C. Pitcher
Hereditary persistence of fetal haemoglobin	(a)	British Royal Infirmary, Sunderland, U.K.	Dr. R. Cartner
	(b)	African The Childrens' Hospital Birmingham, U.K.	Dr. J.R. Mann
Infant blood		MRC Outpatients, Fajara, The Gambia	Dr. E.M. Spalding
High oxygen affinity (Hb Radcliffe)		Radcliffe Infirmary, Oxford, U.K.	Dr. S.T.E. Callender
β thalassaemia trait	(a)	Radcliffe Infirmary, Oxford, U.K.	Dr. S.T.E. Callender
	(b)	Guy's Hospital, London, U.K.	Dr. M.E. Pembrey
Iron deficient		Radcliffe Infirmary, Oxford	Prof. D.J. Weatherall
Sickle cell anaemia (trait and disease)		MRC Outpatients, Fajara, The Gambia	Dr. C. Jones Dr. E.M. Spalding
Glucose-6-phosphate dehydrogenase deficiency		MRC Field Station, Kenada, The Gambia	Dr. I.A. McGregor Dr. M.G.M. Rowland Dr. M. Kerr-Muir
Haemoglobins C and E		King's College Hospital Medical School, London, U.K.	Prof. J.M. White

APPENDIX C

Detailed growth data

(Table numbers correspond to those

in the text)

Expt.No.	1				2			
Cell fraction	Whole blood	Top	Middle	Bottom	Whole blood	Top	Middle	Bottom
SR	0	0	0	0	0	0	0	0
LR	3	2	4	3	1	3	2	0
ST	40	39	36	36	31	40	42	49
LT	30	32	29	24	37	26	24	21
IS	11	11	14	16	14	10	10	10
MS	1	2	3	1	1	2	2	1
G	2	1	0	2	1	0	0	0
A	13	13	14	18	15	19	20	19
MGS	3.1	3.2	3.4	3.0	3.1	2.9	2.9	2.9

Table 19 Cells of different ages

Expt. No	1		2		3		4	
Cell type	A	F	A	F	A	F	A	F
SR	0	0	0	0	15	25	0	0
LR	0	0	0	0	27	26	0	2
ST	31	59	31	62	20	31	23	22
LT	61	38	46	32	22	12	30	15
IS	8	3	23	6	14	1	26	6
MS	0	0	0	0	0	0	6	2
G	0	0	0	0	0	0	0	0
A	0	0	0	0	2	5	15	53
MGS	3.8	3.4	3.9	3.4	2.9	2.2	2.9	1.7

Table 20 Cord and adult cells (same cultures)

Expt. No.	1		2		3		4		5		6		7	
Cell type	A	F	A	F	A	F	A	F	A	F	A	F	A	F
SR	0	2	0	0	5	0	0	0	1	1	1	8	0	7
LR	4	13	3	4	0	7	2	9	6	10	17	13	14	16
ST	33	40	45	62	13	27	46	52	27	37	32	46	25	54
LT	34	29	34	14	32	32	29	18	20	14	24	10	42	2
IS	20	9	7	2	10	15	10	3	19	9	6	2	6	0
MS	0	0	1	0	24	2	6	2	9	3	10	3	0	0
G	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A	9	7	10	18	16	17	7	16	18	26	10	18	13	25
MGS	3.4	3.1	3.2	2.7	3.7	3.1	3.4	2.7	3.2	2.5	3.2	2.4	3.0	2.1

Table 21 Cord and adult cells (parallel cultures)

Cell type	A	A	A	F	F	F	F	F
SR	0	4	0	1	0	0	0	1
LR	1	1	2	3	1	4	1	1
ST	14	19	15	42	45	46	27	43
LT	23	31	24	15	14	20	23	11
IS	34	25	23	12	11	5	8	6
MS	23	7	12	4	2	1	6	0
G	0	0	0	0	0	0	0	0
A	15	13	24	23	27	24	35	38
MGS	3.9	3.5	3.3	2.8	2.6	2.6	2.5	2.1

Table 22 Cord and adult cells (parallel cultures)

1		2	
A	F	A	F
8	34	5	42
10	29	36	38
44	25	25	17
28	9	27	1
6	0	7	0
0	0	0	0
0	0	0	0
4	3	0	2
3.0	2.0	3.0	1.7

Table 23 $\beta^{o}/\delta\beta$ thalassaemia

Expt. No.	1			2			3			4			5			6		
Cell type	A	I	F	A	I	F	A	I	F	A	I	F	A	I	F	A	I	F
SR	0	4	4	0	0	0	0	1	1	4	4	5	3	14	26	21	45	46
LR	10	13	22	0	8	10	6	8	17	8	21	21	21	26	10	5	14	5
ST	34	45	42	45	58	58	29	37	32	22	35	37	31	28	27	4	4	8
LT	52	37	31	45	24	17	19	18	14	38	26	21	34	20	18	18	11	17
IS	2	0	0	6	0	2	20	9	5	25	9	5	8	6	1	30	20	13
MS	0	1	0	0	0	0	2	1	0	1	0	0	0	0	0	19	3	7
G	2	0	1	0	0	0	0	0	0	0	0	0	1	0	0	2	0	0
A	0	0	0	4	10	13	24	26	31	2	5	11	2	6	18	1	3	4
MGS	3.5	3.2	3.0	3.5	2.9	2.7	2.9	2.5	2.1	3.7	3.0	2.7	3.1	2.6	2.0	3.9	2.5	2.5

Table 24 British form of HPFH

Expt No	1		2		2			1			2						3					
Cell type	A	F	A	F	A	F		A	I	F	A	I	F	A	I	F	A	I	F	A	I	F
SR	0	0	1	1	0	0		10	30	28	16	3	12	1	2	2	20	22	24	15	22	30
LR	3	4	6	10	2	4		31	30	34	32	29	31	0	14	4	43	68	67	20	36	28
ST	45	54	27	33	46	44		33	28	28	21	34	33	7	29	42	14	8	7	40	22	22
LT	34	24	20	20	29	23		12	8	4	17	29	22	25	26	22	9	0	2	16	8	4
IS	7	1	19	9	10	4		9	0	0	11	4	2	22	6	14	11	2	0	6	2	2
MS	1	0	9	3	6	4		0	0	0	3	1	0	44	20	10	3	0	0	1	0	0
G	0	1	0	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A	10	16	18	24	7	21		5	4	6	0	0	0	1	3	6	0	0	0	2	10	14
MGS	3.7	3.2	3.2	2.6	3.4	2.8		2.6	2.1	2.0	2.8	3.1	2.7	5.0	3.7	3.5	2.6	1.9	1.9	2.8	2.0	1.8

Table 25 African form of HPFH

Table 26 Young infants

Expt. No.	1		2		3	
Conditions	Aer	An	Aer	An	Aer	An
SR	0	0	7	2	0	0
LR	6	3	58	21	11	3
ST	26	14	24	27	33	15
LT	60	64	5	42	43	51
IS	1	15	0	4	2	22
MS	1	2	0	0	0	7
G	0	0	0	2	0	0
A	6	2	6	2	11	2
MGS	3.4	3.9	2.2	3.2	2.8	4.0

Table 28 Aerobic and low oxygen tensions

	1		2		3		4		5		6	
Cells	C	T	C	T	C	T	C	T	C	T	C	T
	1	0	0	0	1	0	0	1	0	0	5	0
	17	6	3	2	6	10	4	8	6	6	0	5
	32	43	45	44	27	32	33	45	51	70	13	35
	24	24	34	25	20	10	34	29	21	6	32	19
	6	14	7	7	19	14	20	1	8	0	10	5
	10	4	1	1	9	14	0	2	6	0	24	8
	0	0	0	0	0	0	0	2	0	0	0	1
	10	9	10	11	18	20	9	12	8	18	16	27
	3.2	3.3	3.2	3.2	3.2	3.1	3.4	2.9	3.3	2.5	3.7	2.7

Table 30 Haemoglobin with high oxygen affinity (Hb Radcliffe)

Expt. No.	1												2											
Condi-tions	Aerobic				Low O_2				Plunge				Aerobic				Low O_2				Plunge			
Cell type	A	A	F	F	A	A	F	F	A	A	F	F	A	A	F	F	A	A	F	F	A	A	F	F
SR	0	0	0	2	0	0	0	18	0	0	1	5	0	0	0	0	0	0	0	0	0	0	0	0
LR	3	3	4	8	0	1	3	12	7	0	5	11	9	2	10	11	2	0	5	5	3	1	5	4
ST	21	23	40	30	12	29	15	6	18	16	19	21	19	24	21	21	20	9	23	21	18	25	33	37
LT	29	37	17	20	17	26	21	0	33	32	26	22	31	42	22	22	22	22	25	23	43	42	25	23
IS	14	12	5	3	18	9	18	0	7	13	5	6	11	11	1	6	15	21	11	12	6	11	1	2
MS	28	19	2	4	50	32	19	0	30	27	1	6	20	8	2	6	38	42	21	32	18	5	2	1
G	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A	5	6	32	33	3	3	24	64	5	12	43	29	10	13	44	34	3	6	15	7	12	16	34	33
MGS	4.2	4.0	2.4	2.3	4.9	4.3	3.4	0.6	4.2	4.0	2.0	2.4	3.7	3.5	1.9	2.6	4.6	4.7	3.6	4.2	3.7	3.3	2.3	2.3

Table 31 Cord cells (varying oxygen tensions)

Expt. No.	1		2		3		4		5		6		7	
Cell Type	C	T	C	T	C	T	C	T	C	T	C	T	C	T
SR	0	3	0	0	0	0	1	0	1	0	0	0	5	0
LR	6	6	3	2	4	5	17	4	6	4	2	1	0	1
ST	51	59	45	49	33	33	32	49	27	31	46	42	13	17
LT	21	17	34	35	34	43	24	21	20	23	29	28	32	33
IS	8	3	7	7	20	12	6	8	19	17	10	16	10	19
MS	6	3	1	0	0	1	10	8	9	10	8	10	24	21
G	0	0	0	1	0	0	0	0	0	0	0	0	0	0
A	8	9	10	6	9	6	10	10	18	15	5	3	16	9
MGS	3.3	2.9	3.2	3.3	3.4	3.5	3.2	3.3	3.2	3.4	3.6	3.8	3.7	4.1

Table 34 β thalassaemia trait

Expt. No	1		2	
Cell type	C	T	C	T
SR	0	0	1	0
LR	6	8	17	11
ST	51	50	32	34
LT	21	25	24	26
LS	8	4	6	12
MS	6	4	10	9
G	0	0	0	0
A	8	9	10	8
MGS	3.3	3.1	3.2	3.4

Table 35 Iron deficiency anaemia

Expt. No.	1			2			3			4			5			6				7		8		9	
Cell type	AA	AS	AS	AA	AS	AS	AA	AS	AS	AA	AS	AS	AA	AS	AS	AA	AA	AS	AS	AA	AS	AA	AS	AA	AS
SR	3	3	3	0	0	0	0	0	0	0	0	3	1	12	10	0	0	0	0	0	0	0	0	9	8
LR	9	10	13	1	1	0	0	0	0	11	6	7	11	16	25	6	5	3	4	9	17	1	0	24	19
ST	50	44	51	18	24	37	2	0	2	47	35	51	36	46	33	40	47	42	44	46	50	10	11	36	45
LT	18	18	13	65	56	46	10	10	11	24	41	10	28	7	12	31	24	26	23	39	23	22	24	26	21
IS	3	7	4	10	15	12	10	10	9	4	2	1	0	1	0	10	9	11	5	0	1	11	27	4	2
MS	0	6	4	4	1	1	72	75	73	0	0	0	0	0	0	0	0	0	0	1	0	50	32	0	0
G	3	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
A	14	11	11	2	3	4	6	5	5	14	16	28	24	18	20	13	15	18	24	5	8	6	6	1	5
MGS	2.7	3.0	2.8	3.9	3.8	3.6	5.3	5.4	5.3	2.8	2.9	2.2	2.4	2.2	2.1	3.1	3.0	2.9	2.6	3.2	2.8	4.8	4.6	2.9	2.8

Table 36a Sickle heterozygotes (aerobic conditions)

Expt. No.	lO (42 h)			lO (54 h)		
Cell type	AA	AA	AS	AA	AA	AS
SR	O	O	2	O	O	O
LR	8	12	22	3	3	3
ST	37	46	37	21	23	24
LT	46	29	29	29	37	32
IS	2	1	4	14	12	11
MS	O	O	1	28	19	25
G	O	O	O	O	O	O
A	7	12	5	5	6	5
MGS	3.2	2.8	3.O	4.2	4.O	4.1

Table 36a (cont) Sickle heterozygotes, aerobic conditions

Expt. No.	1			2			4			6			8		9		10 (42 h)			10 (54 h)		
Cell type	AA	AS	AS	AA	AS	AS	AA	AS	AS	AA	AS	AS	AA	AS	AA	AS	AA	AA	AS	AA	AA	AS
SR	0	0	0	0	26	6	6	40	25	0	11	5	0	0	0	12	0	3	9	0	0	11
LR	8	0	0	0	0	0	34	17	1	2	31	5	0	0	19	33	21	29	1	0	1	0
ST	45	0	0	14	0	0	32	4	0	16	5	13	6	0	31	38	30	31	0	12	29	0
LT	25	0	0	59	0	0	0	0	0	45	0	0	11	0	43	9	39	23	0	17	26	0
IS	7	0	0	15	0	0	0	0	0	23	0	0	21	0	5	2	6	5	0	18	9	0
MS	0	0	0	10	0	0	0	0	0	11	0	0	59	0	1	0	0	0	0	50	32	0
G	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A	14	100	100	2	74	94	28	39	74	3	46	77	3	100	1	6	4	9	90	3	3	89
MGS	2.9	0	0	4.1	0.3	0.1	1.7	0.9	0.3	4.1	1.0	0.4	5.2	0	3.4	2.4	3.2	2.7	0.1	4.9	4.0	0.1

Table 36b Sickle heterozygotes (low oxygen tensions).

Expt. No.	2			10		
Cell type	AA	AS	AS	AA	AA	AS
SR	0	0	0	0	0	2
LR	2	2	8	7	0	8
ST	28	28	50	18	16	27
LT	27	37	13	33	32	24
IS	9	16	3	7	13	6
MS	24	7	3	30	27	12
G	0	0	0	0	0	0
A	10	10	23	5	12	21
MGS	3.9	3.4	2.5	4.2	4.0	3.0

Table 36c Sickle heterozygotes (plunge)

1		2			3		4		5	
AA	SS	AA	SS	SS	AA	SS	AA	SS	AA	SS
0	5	3	0	3	14	22	0	0	0	0
14	14	9	37	23	14	18	0	4	2	3
24	53	50	28	32	39	30	21	30	12	15
42	20	18	7	12	27	21	43	32	16	25
6	1	3	4	5	2	3	24	10	14	10
0	0	0	3	4	0	0	11	16	51	43
0	0	3	2	1	0	0	0	0	0	0
14	7	14	19	20	4	6	1	8	5	4
3.0	2.8	2.7	2.3	2.4	2.8	2.5	4.2	3.7	4.8	4.6

Table 37a Sickle homozygotes (aerobic conditions)

Expt. No.	3		4		5			1			2			3			4		5	
Cell type	AA	SS	AA	SS	AA	SS		C	T	T	C	T	T	C	T	T	C	T	C	T
ST	0	71	0	50	0	32		0	0	0	1	7	18	0	0	0	0	0	0	1
LR	19	22	4	14	1	39		11	9	18	11	24	21	6	2	0	10	9	3	3
ST	31	2	14	6	4	7		47	50	51	36	29	13	40	55	43	58	70	37	49
LT	43	0	35	0	17	1		24	16	9	28	10	2	31	22	14	23	8	24	7
IS	5	0	22	0	10	1		4	1	1	0	1	0	10	4	2	2	0	15	3
MS	1	0	20	0	68	0		0	0	0	0	0	0	0	0	0	0	0	11	4
G	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	2
A	1	5	5	30	0	20		14	24	21	24	29	46	13	17	41	7	13	10	31
MGS	3.3	1.2	4.3	1.0	5.4	1.4		2.8	2.4	2.3	2.4	1.9	1.1	3.1	2.8	2.0	3.0	2.7	3.5	2.3

Table 37b Sickle homozygotes

(low oxygen tensions)

Table 39 Glucose-6-phosphate dehydrogenase deficiency

Table 38 overleaf

Expt. No.	2			10		
Cell type	AA	AS	AS	AA	AA	AS
SR	0	0	0	0	0	2
LR	2	2	8	7	0	8
ST	28	28	50	15	16	27
LT	27	37	13	33	32	24
IS	9	16	3	7	13	6
MS	24	7	3	30	27	12
G	0	0	0	0	0	0
A	10	10	23	5	12	21
MGS	3.9	3.4	2.5	4.2	4.0	3.0

Table 38 Sickle heterozygotes (plunge)

Expt. No.	1		2		3			4		
Cell type	AA	AC	AA	AC	AA	AA	AC	AA	AA	AC
SR	0	0	7	5	0	0	0	0	0	0
LR	0	0	12	20	5	0	4	2	3	1
ST	6	5	35	35	21	21	20	12	8	8
LT	11	11	29	29	35	43	43	16	23	23
IS	21	17	9	4	24	24	16	14	12	11
MS	59	61	1	0	14	11	15	51	48	54
G	0	0	0	0	0	0	0	0	0	0
A	3	6	7	7	1	1	2	5	6	3
MGS	5.2	5.1	3.0	2.9	4.2	4.2	4.1	4.8	4.7	5.0

Table 40 Haemoglobin C

	1				2				3				4		
	AA	AA	AE	AE	AA	AA	AE	AE	AA	AA	AE	AE	AE	AE	AE
SR	14	13	4	10	0	0	0	0	0	0	0	0	0	0	0
LR	14	17	12	17	5	3	3	2	2	3	1	2	1	0	0
ST	39	39	53	42	21	17	23	22	12	8	4	11	10	8	10
LT	27	24	24	22	35	42	35	46	16	23	20	25	22	22	21
IS	2	1	3	2	24	26	19	11	14	12	19	14	11	15	16
MS	0	0	0	0	14	11	17	18	51	48	54	43	50	50	49
G	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A	4	6	4	7	1	1	3	1	5	6	2	5	6	5	4
MGS	2.8	2.7	3.0	2.7	4.2	4.2	4.1	4.2	4.8	4.7	5.1	4.7	4.8	4.9	4.9

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d.	Large trophozoite	LT	
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FETAL HÆMOGLOBIN AND MALARIA

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Summary The distribution and growth of *Plasmodium falciparum* was compared in red blood-cells containing either adult or fetal hæmoglobins. In in-vitro cultures, cord blood-cells were invaded more readily, but there was a paucity of parasites in cells containing hæmoglobin F in the blood of infected infants aged 3–6 months. These findings suggest that *P. falciparum* may preferentially invade young, metabolically active erythrocytes. There was a significant retardation of parasite growth in vitro in cells containing hæmoglobin F. This latter finding suggests a further mechanism for the resistance to malaria in the first months of life and for high gene frequencies (in areas in which malaria was endemic) of the thalassæmias and related hæmoglobinopathies in which the rate of decline of fetal-hæmoglobin production is retarded.

Introduction

NEWBORN infants have considerable resistance to infection with *Plasmodium falciparum*.[1–3] Explanations for this phenomenon have included immunity acquired from the mother,[4] *p*-aminobenzoic-acid deficiency due to a milk diet,[5] and the high concentrations of fetal hæmoglobin in the erythrocytes during the first few months of life.[2 3 6] Probably more than one mechanism is involved.

The possibility that erythrocytes containing fetal hæmoglobin (HbF) are more resistant to infection with *P. falciparum* than those containing adult hæmoglobin (HbA) has not been explored at the cellular level. This possibility is potentially important because, in addition to suggesting a mechanism for neonatal resistance to malaria, it indicates an approach to the unsolved question of why the thalassæmias are so common in areas where malaria was endemic.[7] HbF production over the first year of life declines more slowly in infants who are heterozygous for β thalassæmia than in normal infants.[8] If HbF confers resistance to *P. falciparum* such infants might be at an advantage in a malarious area, particularly during the critical stage of development when passive immunity to malaria is waning but active immunisation has not yet occurred.[9]

We approached this problem in two ways. First we looked at the relative distribution and growth of *P. falciparum* in fetal and adult cells in tissue-culture; secondly we examined the red blood-cells of infants with *P. falciparum* infections to see whether there is any difference in the distribution of parasites between red blood-cells containing predominantly HbA as compared with HbF. The results indicate that there are significant differences in both the intracellular distribution and growth of *P. falciparum* in HbF as compared with HbA containing cells.

Methods

In-vitro Cultures

Blood infected with synchronous late ring stages of *P. falciparum* was obtained by venepuncture of Gambian children with malaria. The heparinised blood (17 I.U. heparin/ml) was cultured directly in microtissue trays. Microcultures were carried out in duplicate; each well contained 10 µl of blood (infected and fetal) plus 150 µl supplemented medium 199 and 50–100 µl of serum from the donor of the infected cells or AB serum.[10 11] The susceptibility of HbF-containing cells to infection was tested by mixing group-O washed umbilical-cord blood-cells with an equal volume of malarious blood, the cells of which contained mainly HbA. Culture trays were incubated, without shaking, at 37°C in a 5% carbon-dioxide air mixture. The extent of multiplication and subsequent growth of the parasites was assessed by morphological examination of cells recovered from the tissue-culture trays. Thin smears were stained with Giemsa for parasite morphology or treated by a modification[12] of the acid-elution method[13] for demonstrating HbF and parasites. Blood-films treated in this way showed two cell populations; fully eluted "ghosts" which had lost HbA and deeply staining cells which had retained HbF (fetal cells). The relative number of parasites in the "ghosts" and fetal cells was determined by counting all cells in randomly chosen fields.

Distribution of Parasites in Cells Containing HbA and HbF

Blood-samples were obtained by venepuncture from infants aged 3 to 6 months with *P. falciparum* infections. Films were spread and stained with Giemsa or treated by the acid-elution technique. The relative distribution of parasites in cells containing predominantly HbA ("ghosts"), cells containing both HbF and HbA (intermediate cells), and fetal cells was determined. To assess the validity of this visual subdivision of cells the preparations were also examined using a microdensitometer (Watson).[14] Several fields from each preparation were examined and cells designated as "ghost", intermediate, or fetal before their optical densities (O.D.) were read. In addition the O.D. of cells with and without parasites was determined in several acid-elution preparations.

Results

In-vitro Parasitisation of HbF and HbA containing Cells

The data shown in table I are from two experiments in which cultured trophozoites in HbA-containing cells grew in vitro during a period of 70 hours, through schizogony to invasion of new fetal or adult cells, to a

TABLE I—PARASITE MULTIPLICATION IN VITRO IN ADULT "GHOST" (G) AS COMPARED WITH FETAL (F) CELLS

—	Cell type and ratio					
	Experiment 1			Experiment 2		
	G	F	F/G	G	F	F/G
Number of cells examined for second-generation parasites	1183	990	0·8	703	409	0·6
Parasite multiplication	3·7	8·5	2·3	8·8	22·8	2·6
Percentage cells parasitised	4·1	7·4	1·8	3·1	6·9	2·2
Mean number of parasites/infected cells	1·3	1·6	1·2	1·6	1·9	1·2
Mean number of parasites/multiply-infected cell	2·4	2·5	1·1	2·8	2·9	1·0

Parasite multiplication=Parasites/10^2 "ghost" (or fetal) R.B.C. after schizogony/parasites/10^2 "ghost"+fetal R.B.C. before schizogony.

R.B.C.=red blood-cell.

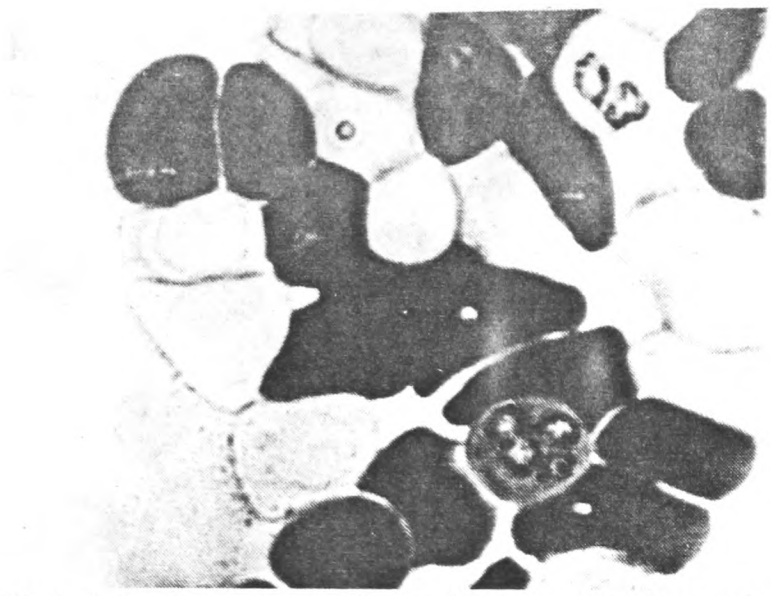


Fig. 1—Acid-elution preparation showing distribution of *P. falciparum* in fetal and adult "ghost" cells in vitro.

Reduced by ⅓ from ×1500.

second generation of young trophozoites (fig. 1). The fetal cells used in both experiments were from the same cord blood-sample. Parasite numbers increased in both experiments with multiplication-rates of 6·6 and 11·2, respectively. During this time the ratio of HbF to HbA containing cells did not change, indicating that differential cell lysis had not occurred. By the end of the culture period parasites were found more than twice as frequently in fetal cells as compared with "ghost" cells. To a large extent this was because almost twice as many HbF-containing cells were infected as compared with those containing HbA, but the average number of parasites in infected cells was also greater in those with HbF. Heparinised cord blood held at 4°C for 36 hours before mixing with subcultured cells was still effective in promoting parasite multiplication.

An experiment designed to examine further the growth of parasites in cells containing HbF or HbA is summarised in table II. The data suggest that the growth of parasites in HbF-containing cells was impaired. To eliminate the effects on growth of parasite competition in multiply-infected cells this analysis was carried out only with cells infected with single parasites.

TABLE II—PARASITE GROWTH IN VITRO IN ADULT "GHOST" (G) AS COMPARED WITH FETAL (F) CELLS

	Cell type and ratio					
	Experiment 1			Experiment 2		
	G	F	F/G	G	F	F/G
Small trophozoite	31	59	1·9	31	62	2·0
Large trophozoite	61	38	0·6	46	32	0·7
Schizont	8	3	0·4	23	6	0·3
Total number of cells counted	100	100	1·0	100	100	1·0

TABLE III—PARASITE DISTRIBUTION IN VIVO BETWEEN HbA-CONTAINING "GHOST" (G) CELLS, CELLS CONTAINING HbA AND HbF (INTERMEDIATE OR I CELLS) AND CELLS CONTAINING MAINLY HbF (F CELLS)

Case no.	Red blood-cell type (%)			Parasitisation of cell-types (%)					$\frac{G}{I}$	$\frac{G}{F}$
				All cells						
	G	I	F	Acid Elution	Giemsa	G	I	F		
1*	65	29	6	13·0	13·0	13·1	13·3	7·6	0·98	1·7
2*	38	54	8	2·0	2·0	2·0	2·1	0·5	0·95	4·0
3	36	44	20	7·0	9·0	9·5	7·8	1·1	1·2	8·6
4	27	38	35	3·7	3·2	6·3	5·1	0·4	1·2	15·8
5	71	21	8	4·5	4·8	5·6	2·2	0·5	2·5	11·2
6	78	16	6	3·8	3·4	4·0	2·8	0·7	1·4	5·7
7	80	15	5	2·0	2·0	2·3	0·8	0·5	2·9	4·6
8	79	18	3	5·2	4·8	5·8	3·2	1·8	1·8	3·2
9*	89	7	4	16·5	17·4	16·4	18·9	12·7	0·9	1·3
10	75	10	15	4·1	4·0	5·0	3·2	0·5	1·6	10·0
11	51	21	28	2·6	2·0	3·7	1·6	1·5	2·3	2·5
12*	43	19	38	2·0	1·6	2·3	2·0	1·5	1·2	1·5
13	53	14	33	2·5	3·2	3·9	2·1	0·6	1·9	6·5
14	87	6	7	16·1	15·1	17·8	6·4	2·9	2·8	6·1
15	46	30	24	7·2	6·3	12·1	4·0	1·7	3·0	7·1
16*	70	16	14	4·3	4·6	4·5	3·7	4·2	1·2	1·1
17	49	19	32	11·7	10·6	17·1	9·1	4·9	1·9	3·5
18	73	14	13	3·8	4·8	4·6	2·8	0·6	1·6	7·7

*Distribution of parasites in different cell types was not statistically significant in these cases.

Indicates a typical experiment

In each case at least 2000 cells were counted. There was good agreement between the parasitisation-rates obtained by the two techniques (table III). Therefore the acid-elution procedure does not result in loss of ring forms, and the presence of deeply stained fetal cells does not prevent the parasites from being identified.

The differential distribution of parasites in fetal as compared with intermediate and "ghost" cells is shown in table IV. In the eighteen samples examined there was a relative paucity of parasites in the fetal and intermediate cell populations. Using the χ^2 test these differences were significant at the 1% level in thirteen cases and not significant in five cases. A typical acid-elution slide from which this data was obtained is shown in fig. 2. In order to confirm these findings the slides were examined using a microdensitometer. To see whether the visual classification into ghost, intermediate, and fetal cells was valid the cells were assigned to one of these groups before their O.D. was determined. Although there is some overlap between the groups, the visual classification is remarkably good (fig. 3). The O.D.s of the parasite-containing cells were then compared with those of the total cell population. The results of a typical experiment are shown in fig. 3 which indicates that the parasites are found predominantly in the cells of lower O.D.—i.e., those containing predominantly HbA. The slight in-

Distribution of parasites between HbF and HbA Containing Cells in Blood of Infants Aged 3–6 Months

The percentage parasitisation of cells was determined using both Giemsa stain and the acid-elution technique.

TABLE IV—PARASITE DISTRIBUTION BETWEEN HbA-CONTAINING "GHOST" (G) CELLS, CELLS CONTAINING HbA AND HbF (INTERMEDIATE OR I CELLS) AND CELLS CONTAINING MAINLY HbF (F CELLS) AFTER RE-INVASION IN VITRO

Case no.	Cell type (%)			Parasite-rate in cell type (%)						$\frac{G}{I}$	$\frac{G}{F}$
				All cells							
	G	I	F	Onset	Time of sample	Multiplication	G	I	F		
10	72	13	15	4·1	6·1	1·5	7·4	2·9	2·4	2·6	3·1
19	31	15	54	0·7	1·8	2·8	4·3	1·6	0·8	2·7	5·4
11	65	20	15	2·7	10·2	3·8	12·2	7·0	5·8	1·7	2·1
12*	55	17	28	2·0	3·3	1·7	3·6	3·6	2·6	1·0	1·4
16*	56	24	20	4·3	2·5	0·6	2·9	1·2	3·2	2·4	0·9
18	66	16	18	3·8	6·1	1·6	7·8	4·0	1·9	2·0	4·1

*Distribution of parasites in different cell types was not statistically significant. In these cases parasite distribution was not significantly different in cell types at onset of culture (table III).

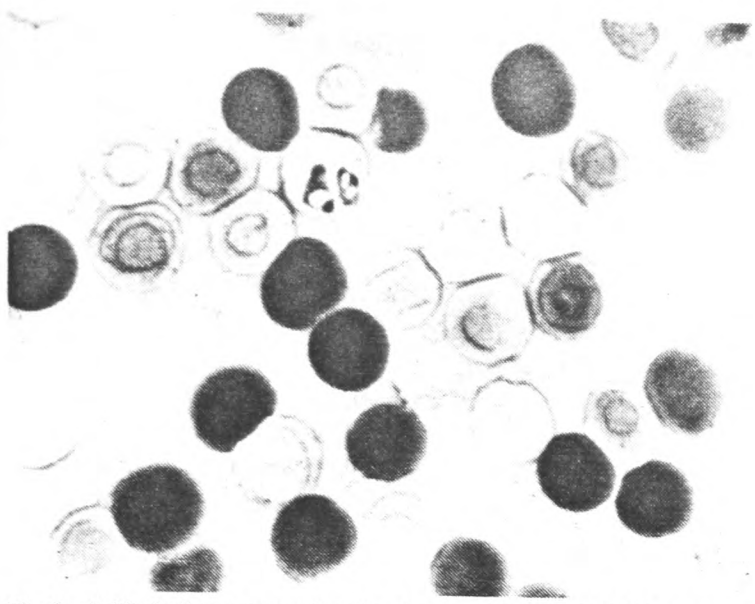


Fig. 2—Acid-elution preparation showing distribution of *P. falciparum* in intermediate and "ghost" cells in the blood of a 3-month-old infant with malaria.

Reduced by ⅓ from ×1000.

crease in the O.D. of the parasitised cells as compared with those of the fully eluted "ghosts" may partly result from an increase in O.D. due to the presence of the parasite.

To see whether the excess of parasites in the HbA-containing cells was due to preferential loss of parasitised HbF-containing cells from the circulation, blood-samples from several of these children were incubated in vitro until the parasites had invaded a new population of red blood-cells. Samples were examined at regular intervals to ensure that all parasites of the first generation had developed beyond the ring form. The distribution of second-generation ring forms is shown in table IV. Again the parasites were found predominantly in the HbA-containing population. In the two cases in which there was no difference in the distribution of parasites between cell-types after reinvasion there had been no significant difference in the original sample.

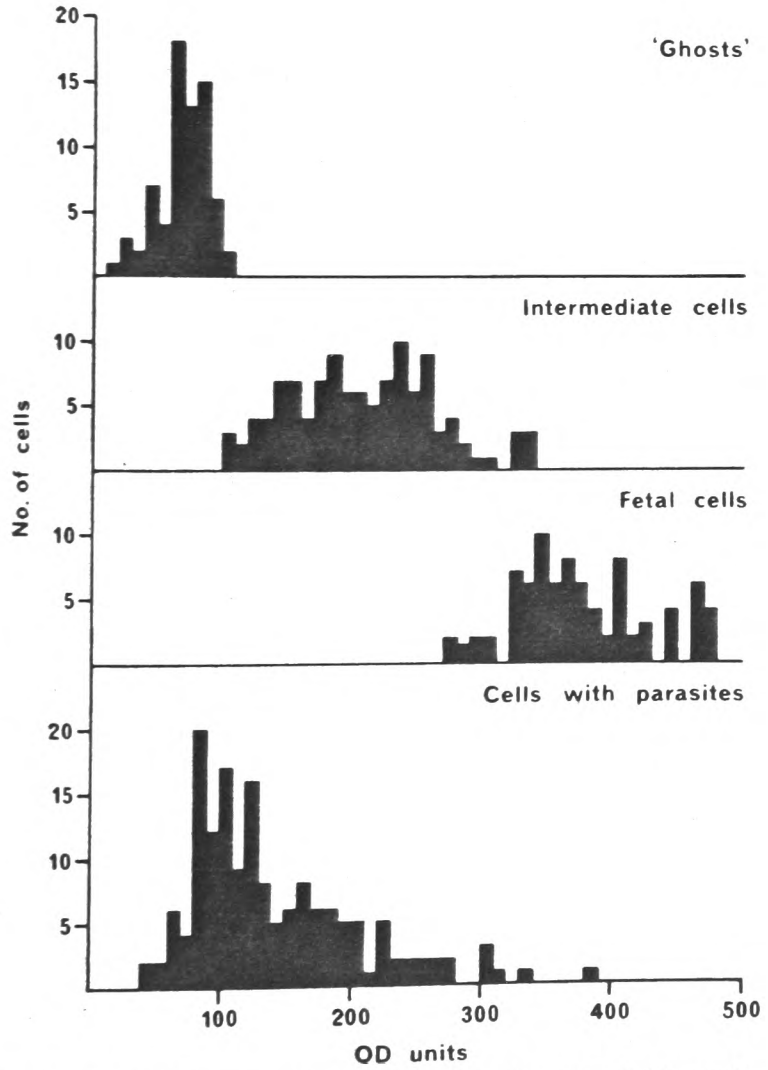


Fig. 3—Acid-elution preparation from blood of a 3-month-old infant with *P. falciparum* malaria studied by microdensitometry.

Top three histograms show O.D.s of "ghost", intermediate, and fetal cells. Fourth histogram shows distribution of O.D.s of cells from the same preparation containing *P. falciparum*.

Discussion

These experiments indicate that there are significant differences in the distribution and growth of *P. falciparum* in fetal as compared with adult erythrocytes. In the in-vitro studies the parasites entered both the fetal and adult cells readily, although the invasion-rate for the fetal cells was higher than that of the adult cells. However, in experiments designed to examine the further development of parasites there was a significant retardation in the rate of development of the parasites in cord blood as compared with in adult cells. Further work is required to determine whether the growth retardation is due to the presence of HbF in the fetal cells or whether it is due to other differences in cell chemistry. *P. falciparum* requires oxygen for its development[15] and it is possible that the high oxygen affinity of HbF results in an environment where oxygen is less available to the parasite.

Studies of parasite distribution between HbF and HbA containing erythrocytes in the blood of infants during the first year of life showed a significant difference between the two cell populations. In most of the experiments there was a paucity of parasites in the HbF-containing population and this was not due to an elution or staining artefact. Clearly such a difference could occur if (1) the HbF-containing cells were not being invaded, (2) the cells were being invaded but the parasite was not developing, (3) the HbF-containing cells and parasites were being preferentially removed from the circulation. In the further set of experiments in which the parasites in the blood of these infants were allowed to develop in vitro and reinvade red blood-cells, an unequal distribution of parasites between HbF and HbA containing populations was also observed. These results rule out preferential removal of HbF-containing parasitised cells as a likely explanation for the unequal distribution and suggest a relative failure of invasion of the HbF-containing cells. It is still possible, however, that the cells were invaded but that the parasites did not develop well enough to be seen on light microscopy although this seems very unlikely. The apparent discrepancy between the relative rates of invasion of HbF-containing cells in vitro as compared with in vivo may reflect the particular ages of the cell populations being studied. Since HbF is replaced by HbA during the first year of life, the HbF-containing cells in young infants represent an older population than those which contain HbA. It is possible that *P. falciparum* invades predominantly young and metabolically active erythrocytes; this would account for the increased invasion-rate of the cord blood-samples studied in vitro and for the paucity in the HbF cells of young infants. This seems to us to be a likely possibility

despite the long-held view that *P. falciparum* invades cells of all ages equally.[16] Further experiments to examine this possibility are under way. Because of the different turnover-rates of cells containing HbA as compared with HbF in the blood of thalassæmic patients,[7] this problem must be resolved before studies of the type we describe can be applied directly to patients with this disorder.

It appears, therefore, that there are clear differences in the behaviour of *P. falciparum* in HbF-containing as compared with HbA-containing red-blood-cell populations, both in vitro and in vivo. Further studies are required to see whether these differences also occur in adult erythrocytes containing HbF and HbA and whether they are related primarily to invasion, cell age, or to development, or to more than one mechanism. However, assuming that the rate of decline of HbF production is retarded in the first year in infants with thalassæmia or other hæmoglobinopathies, the findings outlined here offer further possible mechanisms for the maintenance of the high gene frequencies of some of these disorders.

We thank Dr R. S. Bray and his colleagues at the Medical Research Council Laboratories, Fajara, Gambia for their help and hospitality, and Mr A. K. Rahman and Dr E. A. Spalding for the collection of specimens. This work was supported by the Medical Research Council.

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Hypothesis

INSULIN RATHER THAN GLUCOSE HOMŒOSTASIS IN THE PATHOPHYSIOLOGY OF DIABETES

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Summary Hepatic glucose efflux is very sensitive to changes in plasma-insulin and it is postulated that this provides an important feedback control of insulin secretion. In diabetes this hepatic "insulin sensor" increases the basal plasma-glucose until the impaired β cells are sufficiently stimulated to secrete normal basal insulin concentrations. Glucose regulation thus becomes of secondary importance to the maintenance of basal insulin secretion, which is teleologically needed for the "anabolic" requirements of cell growth. This hypothesis provides an explanation for the "normal" basal plasma-insulin concentrations found in diabetes in spite of impaired β-cell function. This maintenance of basal insulin secretion accounts for the discrepancy between the marked hyperglycæmia and minimal ketosis of maturity onset diabetic patients.

ROLE OF INSULIN

INSULIN is secreted in response to an animal's main nutrients and has been termed the "hormone of nutrient storage."[1] Thus insulin secretion following meals promotes "anabolism", and the diminished insulin secretion of starvation assists "catabolism" with release of stored nutrients.[2] A further anabolic role of insulin is the stimulation of several synthetic functions of cells, which collectively have been termed the "pleiotypic response".[3] Thus insulin enhances cell growth in tissue-culture.[4] Normally nutrient regulation and stimulation of cell growth are closely interlinked. This need not always be the case, and in the fetus insulin secretion is probably related to cell growth, with the fetal nutrient concentration being to a large extent controlled by the maternal nutrient regulation.[5]

Insulin deficiency results in diabetes. The observed high plasma-glucose is usually regarded as the simple, inevitable result of diminished insulin secretion, signifying a failure of nutrient regulation. However, in diabetes the plasma-glucose concentration is raised even in the fasting state, and during the night remains remarkably steady.[6] In mild diabetes the plasma-glucose is proportionately more abnormal in the basal state than after a meal.[7]

We postulate that the basal plasma-insulin concentration needs to be kept above a certain minimum value for normal cell function, and that teleologically the insulin requirement for cell growth is more vital than strict nutrient regulation. In diabetes we suggest that the high plasma-glucose provides a very necessary positive feedback which sufficiently stimulates the impaired β cells to produce normal fasting insulin concentrations. If these are maintained, excessive nutrient catabolism, including aminoacid release from muscles and lipolysis, is prevented.

BASAL INSULIN SECRETION IN DIABETES

Diabetic patients have an impaired insulin secretory response to a rise in plasma-glucose, yet have fasting basal plasma-insulin concentrations similar to those of normal people.[7–12] Even severely diabetic, ketotic patients have peripheral basal plasma-insulin concentrations which are only half the normal concentration.[13] This constancy of basal insulin secretion is one of the most remarkable facets of diabetes.

Porte et al. have postulated that there are two modes of insulin release, one for basal and one for stimulated insulin secretion.[12 14] Two lines of investigation have been thought to support this view. Firstly, the insulin response to an infusion of glucose, which only slightly raises the "basal" plasma-glucose, has quite different

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Differentiation of gametocytes of *Plasmodium falciparum* *in vitro*

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In a few microcultures of *P. falciparum* (Phillips, Rahman and Wilson, 1975), gametocytes differentiated from the ring stage parasites with which the cultures were initiated. The infected blood came from Gambian children in September and October (1973 and 1975). Relatively few rings differentiated into gametocytes which were first morphologically recognizable after 40-50 hours of culture when the majority of rings had grown into schizonts and reinvasion was in progress. Multiple chromatin-like granules were apparent in immature gametocytes by 55-65 hours and the crescent form was developing. In two cultures the gametocytes appeared to differentiate from ring stages which were the products of schizogony *in vitro*. This was confirmed by adding cord blood at the start of some cultures and finding gametocytes in foetal cells 50-60 hours after schizogony and reinvasion had occurred in the culture.

Phillips, R.S. *et al*, 1975. Trans.roy.Soc.trop.Med.Hyg. 69, 432.

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Bulletin of the World Health Organization, 55 (2-3): 179-186 (1977)

Invasion and growth of *Plasmodium falciparum* in different types of human erythrocyte*

R. J. M. WILSON,[1] G. PASVOL,[2] & D. J. WEATHERALL [3]

The susceptibility of human red blood cells to invasion by Plasmodium falciparum *was investigated in microtissue cultures with different populations of erythrocytes containing fetal haemoglobin (HbF). Preferential invasion of HbF-containing erythrocytes was observed with umbilical cord blood. The parasites showed no preference for HbF cells in blood from a subject with hereditary persistence of fetal haemoglobin (HPFH). By contrast, a significant preference for HbA-containing erythrocytes was found with blood from young infants. Further experiments demonstrated that falciparum parasites preferentially invade "young" erythrocytes. This could explain the distribution of parasites found in blood containing HbF, because HbF-containing erythrocytes are "younger" on average in cord blood, "older" in the blood of infants, and of the same average age as HbA-containing cells in HPFH. We concluded that the susceptibility of human erythrocytes to invasion by* P. falciparum *is not correlated with the presence of HbF, but that aging of red cells* in vivo *decreases their susceptibility to invasion. Semi-quantitative measurements were made of parasite growth in cells containing HbA or HbF. The growth of individual parasites in cells containing HbF was significantly retarded. This might confer a selective advantage on individuals with thalassaemia and sickle cell trait, in which HbF levels are raised in early life.*

We have been interested in the possibility that erythrocytes containing fetal haemoglobin (HbF) might engender resistance to malarial infection (*1*, *2*). High frequencies (50–90%) of red cells containing HbF are found in the peripheral circulation at birth. After a few weeks, HbF levels begin to decrease linearly to reach about 5% at 100 days from birth (*3*). There is evidence, however, that red cells containing HbF persist at a higher level for longer periods in infants with β-thalassaemia or sickle cell anaemia (*4*). Proposals that malaria has influenced the gene frequencies of the latter two conditions are well known.

Recently, we reported investigations at the cellular level on the susceptibility of human red cells containing HbF to invasion by *Plasmodium falciparum* (*5*). These studies, together with the further observations summarized in this paper, clearly indicate that the susceptibility of red cells to invasion is related to their age rather than to the presence of HbF. Parasite development, however, is retarded inside erythrocytes containing HbF. Further experimental details of these investigations will be published later (Pasvol et al., in preparation).

MATERIALS AND METHODS

Collection of blood samples

Blood was collected into sterile heparinized tubes and stored at 4°C for up to 24 h if not required immediately. Malarious blood was incubated within 3 h of collection if used for culture. Blood samples from the United Kingdom were transported on ice to the Gambia and cultures were set up within 30 h of venepuncture.

Infected blood. Blood containing late, synchronous ring-stage *P. falciparum* parasites was obtained from patients aged 2–5 years who attended the outpatients clinic at the British Medical Research Council Laboratories, Fajara, the Gambia.

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Infant blood. Blood with different numbers of HbF-containing erythrocytes was obtained from Gambian children under the age of 6 months.

Cord blood. Blood containing 90–95% cells with demonstrable HbF was collected from the umbilical cord of healthy Gambian newborn babies at the Royal Victoria Hospital, Banjul, the Gambia.

Hereditary persistence of fetal haemoglobin (HPFH) blood. Blood was collected from a family known to be haematologically normal except that they have about 18% HbF unevenly distributed among their red cells; the cell populations that contain mainly HbF or HbA have similar rates of turnover and are therefore made up of cells of a similar metabolic age (*6*).

Invasion of reticulocytes

Red cells from patients suffering from falciparum malaria were stained for reticulocytes with brilliant cresyl blue and counterstained for parasites with Giemsa's stain. The relative rates of invasion of reticulocytes and nonreticulocytes was assessed by counting the number of parasites per 1000 cells of each type.

Cell separation by age using centrifugation

Packed red cells from which the buffy coat had been removed were centrifuged in a Wintrobe tube at 4°C and 2500 *g* for 1 hour. With a Pasteur pipette, 50-μl samples were removed from the top, middle, and bottom of the tubes. Reticulocyte counts were carried out on each fraction to assess the degree of separation of cells by age and the percentage parasitization of each fraction was determined.

Intracellular distribution of HbF and malarial parasites

Blood smears were treated by a modification of the acid-elution technique (*7*), which elutes HbA but not HbF from red cells and which previous studies have shown does not elute parasites (*5*).

Infection of cells with P. falciparum in vitro

To examine the invasion of red cells by *P. falciparum*, uninfected " recipient " and infected " donor" cells were mixed and maintained for up to 3 days under tissue culture conditions (*8*). The red cells (either from whole blood or after fractionation according to age) were washed three times under sterile conditions with modified Ringer's solution (*9*). Donor and recipient cells were mixed in various proportions; details are given below. Between 5 and 10 μl of mixed cells were suspended in an incubation medium consisting of 4 volumes of supplemented Medium 199 and 1 volume of human AB serum, and dispensed into flat-bottomed microtissue culture trays with a volume of 250 μl per well. The trays were incubated without shaking at 37°C in an atmosphere of 5% CO_2 and 95% air. Under these conditions, the parasites in the infected cells matured into schizonts and released a new generation of merozoites, which invaded the uninfected cells present in the culture. The multiplication of parasites *in vitro* was calculated by dividing the percentage of parasites following reinvasion by the percentage present in the initial mixture, after counting 1000 cells in each case.

Growth measurements

Infected HbA- and HbF-containing cells were distinguished in smears by acid elution. Giemsa-stained parasites were photographed on 35-mm film (Recordak " Microfile ") at a magnification of approximately × 530. Infected cells were photographed at random in successive microscopic fields, except that cells containing only a single parasite were selected. Negative images were enlarged 10 times and the parasite outlines were traced on graph paper. Relative growth measurements were made from cutout, weighed images or from area computations.

RESULTS

Invasion of red cells containing HbF

Blood from infected infants. Blood films were made from 18 infected infants aged 3–6 months. Cells containing HbF were identified by the acid-elution method. The relative distribution of parasites in cells containing predominantly HbA (ghosts), in cells containing both HbA and HbF (intermediate cells), and in fetal cells was determined. In five cases, the distribution of parasites in different cell types could not be distinguished statistically. In the remainder (see Fig. 1), the parasites were found predominantly in cells containing HbA (χ^2 test gave $P < 0.01$).

To determine whether the excess of parasites in HbA-containing cells was due to a preferential loss of parasitized HbF-containing cells from the circulation, blood samples from six infected infants were cultured *in vitro* to allow the parasites to invade a new population of red cells. In two of the cases, the parasites were distributed equally between

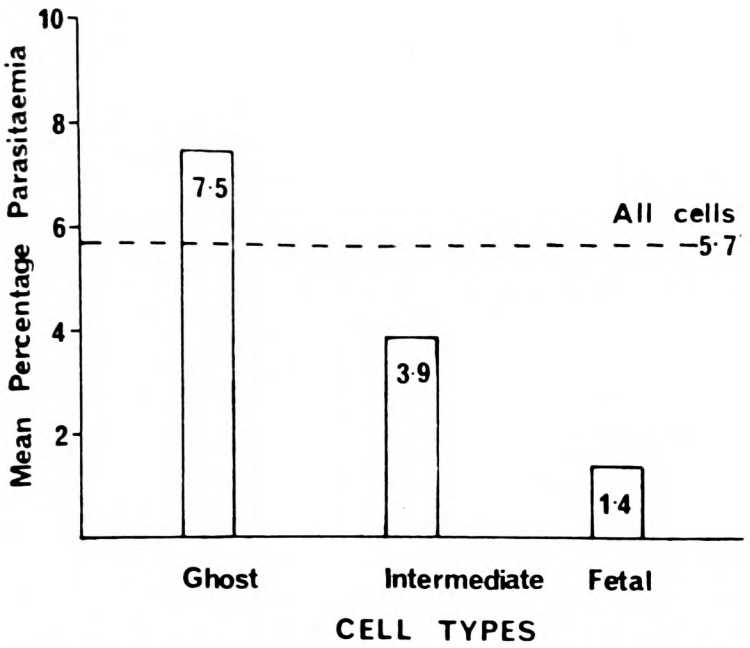


Fig. 1. The distribution of parasites in erythrocytes containing HbA (ghost) or HbF, in blood smears from 13 infants infected with *P. falciparum*.

the different cell types, both at the onset and after completion of the cultures. The distribution of the second generation ring-stage parasites in different red cell types in the other four cultures is shown in Fig. 2. In these instances, the distribution of parasites between HbA- and HbF-containing cells was essentially unchanged after reinvasion, i.e., parasites were still found predominantly in erythrocytes containing HbA. The preference of parasites for red cells containing HbA could not be accounted for by a differential loss of HbF cells in these cultures.

Umbilical cord blood. A different result was obtained when falciparum parasites were cultured in the presence of HbF-containing cells derived from washed umbilical cord blood. This blood was mixed with an equal volume of infected blood, the cells of which were mainly HbA, and was cultured as above. The results of three experiments are summarized in Fig. 3. After the invasion of new red cells, parasites were more than twice as frequent in cells with HbF than in those containing HbA. Multiple-infected erythrocytes occurred with both types of cell but twice as many cells containing HbF were infected. The ratio of HbF- to HbA-containing cells changed little during the cultures, indicating that differential cell lysis was minimal.

These results indicated that HbF-containing cells in 3–6-month-old infants were markedly less susceptible to malarial infection than HbF-containing cells from umbilical cord blood. This could represent a preference of the parasite for younger, metabolically more active cells because HbF is largely replaced by adult haemoglobin during the first

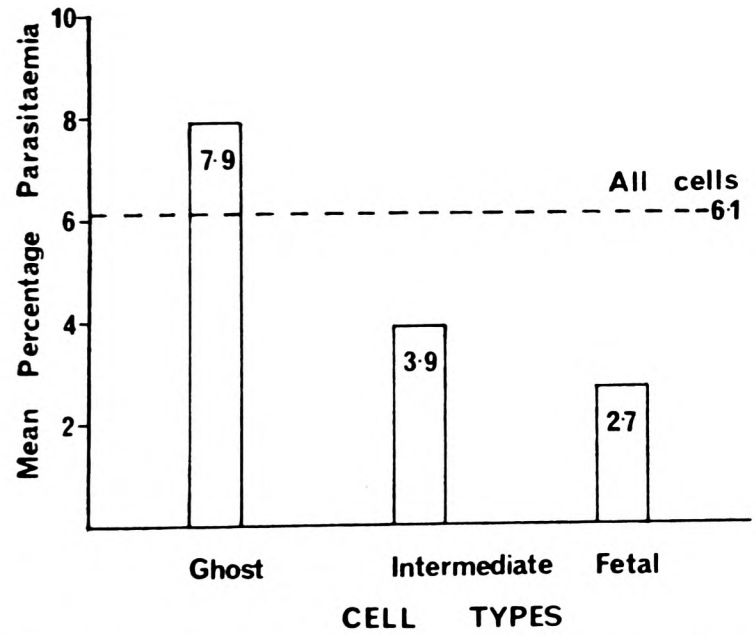


Fig. 2. The distribution of second generation parasites in erythrocytes containing HbA (ghost) or HbF, in tissue cultures of blood from four infants infected with *P. falciparum*.

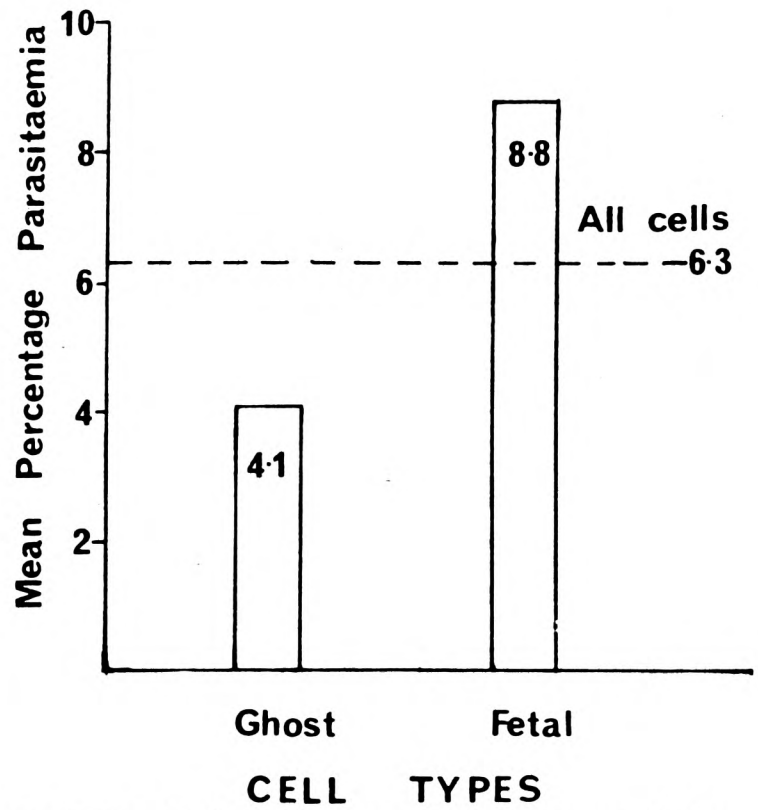


Fig. 3. The distribution of second generation parasites in erythrocytes containing HbA (ghost) or HbF, in tissue cultures in which infected blood (HbA) was mixed with an equal volume of uninfected umbilical cord blood (mostly HbF).

Table 1. Differential rates of invasion of reticulocytes and nonreticulocytes in four patients infected with *P. falciparum*

Case	Parasitaemia (%)	Reticulocytosis (%)	Parasites in 1000 reticulocytes (R)	Parasites in 1000 Nonreticulocytes (NR)	R/NR
1	2.2	3.5	42	15	2.8
2	3.1	1.5	102	25	4.1
3	8.1	8.0	114	72	1.6
4	13.1	1.3	220	110	2.0

6 months of life, with the result that HbF-containing cells in young infants represent an older cell population than those that contain HbA (*10*). To test this hypothesis, we re-examined the question of erythrocyte age and susceptibility to infection with *P. falciparum*. The results presented in the next section of this paper do not support the long-held view that infection of red cells with *P. falciparum* occurs regardless of cell age.

Cell age and susceptibility to infection

The relative rates of infection of reticulocytes and nonreticulocyte erythrocytes in blood films from four infected children are shown in Table 1. In each case, a greater proportion of reticulocytes was parasitized than nonreticulocytes (χ^2 test gave $P < 0.01$). This distribution was confirmed when erythrocytes from infected children with ring-stage parasites were separated according to age by centrifugation in a Wintrobe tube. Table 2 shows reticulocyte counts in samples taken from the top, middle, and bottom of each column of centrifuged erythrocytes, to assess the degree of cell separation by age. Table 2 also shows the percentage parasitization of cells in each fraction. Effective reticulocyte separation was seen in each case and there was a significantly greater number of ring-stage parasites in the top than in the middle or bottom fractions (χ^2 test gave $P < 0.005$).

To investigate the effect of cell age on invasion *in vitro*, red cells from two healthy adults and from the cord blood of a healthy infant were centrifuged as described above. Cells from the top, middle, and bottom fractions of centrifuged blood were mixed with infected blood at a ratio of approximately 10 : 1 and were cultured *in vitro*. The multiplication rate of parasites following invasion of the fractionated cells is shown in Table 3. In each case, the rate of invasion of the younger cells in the top fraction of the centrifuged cells was at least twice as great as in the older cells from the bottom of the tube.

Effect of cell age on susceptibility to invasion of different cell populations containing HbF

The experiments described so far in this paper have shown that HbF-containing cells in cord blood are preferentially invaded but that they are less susceptible in blood from 3–6-month-old infants. We also examined the susceptibility *in vitro* of erythrocytes obtained from an adult with hereditary

Table 2. Separation of infected cells into different age groups by centrifugation

Case No.	Reticulocytes (%)				Parasites (%)				T/B
	Whole blood	Top	Middle	Bottom	Whole blood	Top (T)	Middle	Bottom (B)	
1	0.3	5.7	0.2	0	11.5	30.6	12.8	11.4	2.7
2	1.5	4.7	0.8	0.4	2.2	4.3	2.6	2.5	1.7
3	1.9	4.1	0.7	0.1	13.9	30.1	13.5	3.2	9.4
4	5.0	74.8	2.8	0.5	2.2	4.9	1.3	0.6	8.2
5	8.9	19.9	5.6	1.2	7.5	17.3	6.7	5.0	3.5

Table 3. Multiplication of parasites in cultures containing cells of different ages

Case No.	Multiplication rate				T/B
	Whole blood	Top (T)	Middle	Bottom (B)	
Adult 1	1.90	2.73	1.72	0.98	2.8
Adult 2	2.80	4.30	3.00	2.20	2.0
Cord 1	1.52	1.91	1.27	0.77	2.5

persistence of fetal haemoglobin (HPFH). In this blood, HbF and HbA occur in cells of similar metabolic age (*6*). The results of two experiments in which these cells were mixed with a minimal amount of infected blood ($^1/_6$ to $^1/_{10}$ by volume) are summarized in Table 4, group B. For comparative purposes, Table 4 also gives the range of values found for invasion of HbF-containing cells in cord blood (group A), and in the blood of infants under 6 months of age (group C). In contrast to these other cell populations, the rate of invasion of HPFH cells by the parasite was found not to be associated with the presence of HbF.

Effect of HbF on the growth of intra-erythrocytic parasites

We have previously reported morphological data which suggested that the growth of parasites inside HbF-containing erythrocytes was impaired (*5*). Improved data to support this finding are shown in Fig. 4. This gives semi-quantitative estimates of the growth of 231 individual parasites in singly-

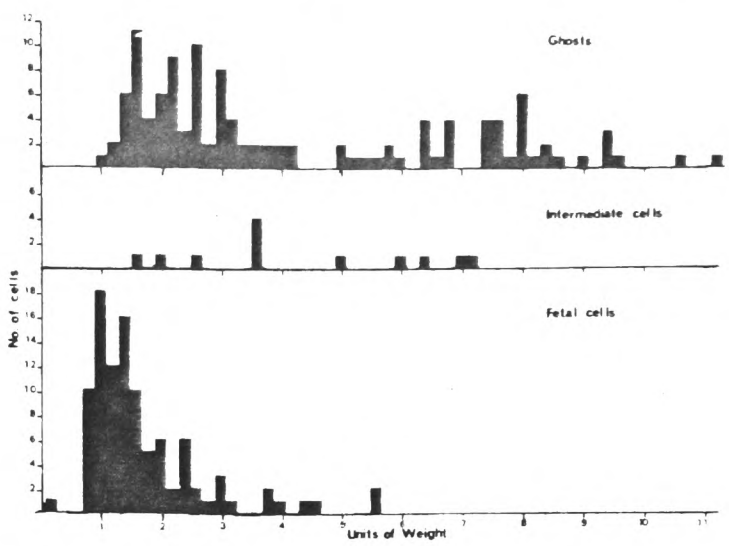


Fig. 4. The 'size' distribution of individual second generation parasites growing in tissue cultures in singly-infected erythrocytes that contained either HbA (119 instances), HbF (100 instances) or an intermediate mixture of HbA and HbF (12 instances). 'Size' measurements were obtained from weighed cutout photographic images.

fnfected erythrocytes from a culture in which ialciparum parasites were allowed to invade a mixture of adult and umbilical cord blood. The frequency distributions of parasite " size " following growth of the second generation parasites show that the development of parasites in fetal cells was significantly retarded compared to that in adult cells. The largest parasites found in HbA-containing cells had reached the stage of large trophozoites and schizonts.

DISCUSSION

Our experiments show that *P. falciparum* invades young human red cells in preference to older ones. This fact alone may explain the distributions of

Table 4. Invasion of HbF-containing cells that are younger (group A), of the same age (group B), or older (group C) than HbA-containing cells in the same culture

Group	Infected blood	Un-infected blood	Red cell type (%)		Parasitization of cell types (%)			G/F
			Ghost	Fetal	All cells	Ghost (G)	Fetal (F)	
A	Adult 1	Cord 1	54	46	8.4	5.2	12.1	0.4
	2	2	52	48	9.3	6.5	12.4	0.5
B	3	British HPFH 1	62	17	9.6	9.6	10.2	0.9
	4	2	41	40	9.3	9.2	9.8	0.9
C	Infant 1	—	65	15	10.2	12.2	5.8	2.1
	2	—	31	54	1.8	4.3	0.8	5.4

parasites that we have found in different populations of red cells containing HbF. In the blood of infected infants under 6 months of age, there was a paucity of parasites in erythrocytes containing HbF; these cells are " older " on the average than their fellow HbA-containing cells. Heavy and preferential parasitization of HbF-containing erythrocytes was observed, on the other hand, when their average age was "younger", as in umbilical cord blood. A British case of HPFH provided a unique naturally occurring control, since there is evidence (*6*) that the cells that contain predominantly HbA or HbF are of similar metabolic age; preferential parasitization of erythrocytes with different types of Hb was not observed in this instance. We conclude that the susceptibility of erythrocytes to invasion by *P. falciparum* is not correlated with the presence of HbF but that the aging of red cells *in vivo* decreases their susceptibility to invasion. Luzzatto et al. (*11*) have also suggested, on different grounds, that *P. falciparum* preferentially invades young red cells in subjects with glucose-6-phosphate dehydrogenase deficiency.

Interaction of falciparum malarial parasites with membrane components that disappear during red cell aging might explain the preferential invasion of erythrocytes. Profound surface reorganization occurs in young erythrocytes during transition from the reticulocyte; surface antigens such as HL-A disappear at this time (*12*). Other studies have shown that old erythrocytes contain 30% less D-galactose residues on their surface than young cells (*13*). The relative resistance of " older " erythrocytes to invasion by *P. falciparum* might provide a temporary protective mechanism against malarial infection in newborn infants. After birth, erythropoiesis shuts off completely and for about a month existing red cells are steadily lost from the circulation as they age. Replacement with new red cells does not occur until haemoglobin levels physiological for the newborn are reached (*3*). The reduced numbers of young, metabolically active red cells in the circulation at this time would confer some degree of protection against fulminating malarial parasitaemias.

In our study, the peripheral blood smears of a few infected infants (5 out of 19) were exceptional in that little difference was seen in the distribution of parasites between cells containing HbA and those containing HbF. This might have resulted from the production for a more prolonged period than usual of red cells that contained HbF. Alternatively, it might indicate the existence of parasites with different invasive capabilities (*14*).

In the investigations of Raper (*15*) and Luzatto (*11*, *16*) on the interaction between falciparum malarial parasites and red cell genetic variants containing HbS or glucose-6-phosphate dehydrogenase deficiency, three possible mechanisms of intrinsic cellular resistance were considered: (1) " failure of infection " due to discrimination at the red cell surface membrane; (2) " abortive infection " due to poor intracellular growth of the parasite; and (3) " suicidal infection " due to the destruction of infected erythrocytes before completion of the parasite's intracellular development. Our investigations have demonstrated that the aging of red cells increases their resistance to infection (mechanism 1). It is perhaps more important that we have strengthened our earlier data (*5*), which suggested that the growth of *P. falciparum* is retarded in cells containing HbF (mechanism 2). The observations illustrated in Fig. 4 were made from parasites growing in fetal cells from umbilical cord blood. In further unpublished work (Pasvol et al., in preparation), we have extended this finding to HbF-containing erythrocytes in the blood of infected infants and subjects with β-thalassaemia or HPFH. Pigment granules are produced in the course of the growth of stunted parasites in HbF-containing erythrocytes, but the way in which HbF exerts its deleterious effect on the parasite is not yet understood. Weatherall (*17*) has suggested that the high affinity of HbF for oxygen might render the parasites relatively hypoxic.

As HbF production persists for longer than usual in the first years of life in infants with thalassaemia and some of the other congenital haemolytic anaemias (*4*), the antiparasitic (growth) effect we have associated with HbF might confer a selective advantage on such children during their early childhood years in malarious areas until protective immunity is acquired. Such an advantage might have helped to maintain the high gene frequencies associated with these disorders in areas where malaria is or has in the past been prevalent.

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RÉSUMÉ

INVASION ET CROISSANCE DE *PLASMODIUM FALCIPARUM* DANS DIFFÉRENTS TYPES D'ÉRYTHROCYTES HUMAINS

Les auteurs ont étudié en cultures tissulaires la sensibilité des érythrocytes humains à l'invasion par *Plasmodium falciparum*, sur différentes populations d'érythrocytes contenant l'hémoglobine fœtale (HbF). Ils ont observé une invasion préférentielle des érythrocytes contenant HbF dans le sang du cordon ombilical. Les parasites n'ont pas manifesté de préférence pour les cellules HbF du sang d'un sujet présentant héréditairement une persistance de l'hémoglobine fœtale (HPFH). En revanche, on a noté une importante préférence pour les érythrocytes HbA du sang des jeunes enfants.

D'autres expériences ont montré que les parasites falciparum envahissaient de préférence les érythrocytes « jeunes ». Ceci pourrait expliquer les répartitions parasitaires observées dans le sang HbF puisque les érythrocytes HbF sont « plus jeunes » en moyenne dans le sang du cordon ombilical, « plus vieux » dans le sang des jeunes enfants, et du même âge moyen que les cellules HbA dans l'HPFH. Les auteurs en ont conclu que la sensibilité des érythrocytes humains à l'invasion par *Plasmodium falciparum* était sans corrélation avec la présence des HbF, mais que le vieillissement des érythrocytes *in vivo* diminuait leur sensibilité à l'invasion.

On a effectué des mesures semi-quantitatives de la croissance des parasites dans les cellules contenant HbA ou HbF. Dans ces dernières, elle était notablement retardée. Ce fait pourrait conférer un avantage sélectif aux individus atteints d'une anémie hémolytique telle que la thalassémie et la drépanocytose, où les taux d'HbF sont augmentés.

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Effects of foetal haemoglobin on susceptibility of red cells to *Plasmodium falciparum*

HIGH gene frequencies for the sickling disorders and β thalassaemia may be due to the relative protection against *Plasmodium falciparum* malaria which has been afforded to heterozygous carriers[1]. In sickling disorders the properties of the abnormal haemoglobin may be responsible for this phenomenon[2–4] but the red cells of adult β thalassaemia heterozygotes contain reduced amounts of normal haemoglobin[5] and are indistinguishable from normal red cells with respect to the rates of invasion and growth of *P. falciparum* (G.P., D.J.W. and R.J.M., in preparation). Hence it is not clear how the β thalassaemia polymorphism has been maintained. There is a possible explanation, however. At birth red cells contain mainly foetal haemoglobin (Hb F); adult haemoglobin (Hb A) replaces Hb F during the first year of life. The rate of decline of Hb F production during this period is retarded in infants heterozygous for β thalassaemia as compared with normal infants[6]. If the presence of Hb F were to protect red cells against *P. falciparum*, β thalassaemia carriers aged 6–18 months might be at an advantage when passive immunity to *P. falciparum* is waning but active immunity is not fully established and mortality from malaria is high[7]. To test this hypothesis we have compared the rates of invasion and growth of *P. falciparum* in red cells containing Hb A with those containing Hb F using a modification of the *in vitro* culture system[8–10].

Samples of 5 ml of blood containing synchronous late ring stages of *P. falciparum* were collected into heparin (10 IU ml^{-1}) and layered onto a 6 × 2 cm column of Whatman CF11 cellulose in phosphate-buffered saline (PBS). The leukocyte-depleted red cells were washed through the column into 2-l Ehrlenmeyer flasks with 100 ml supplemented medium 199[8]. The flasks were gassed with 5% CO_2–95% air and incubated at 37° C with gentle shaking. After about 24 h, when the parasites had grown to the schizont stage, the red cells were recovered by centrifugation and reconstituted to the original 5 ml volume with medium 199. Plasmagel 2.5 ml (3% w/v gelatin; Bellon) was added and the cells were allowed to sediment for 15 min at 37 °C to concentrate the schizont-infected cells in the upper layer. The latter were then suspended in a mixture of 3 parts supplemented medium 199 and 1 part non-immune AB serum. The uninfected red cells which were to be examined for the rate of parasite invasion and growth were collected into heparin, washed three times in PBS, the buffy coat removed and added to an appropriate volume of the parasitised red cell suspension so that the final concentration was approximately 80 × 10^3 cells per μl with 2–5% schizont-infected cells. The mixture was plated into the wells of a microtissue culture plate (250 μl per well) and incubated in 5% CO_2–air at 37 °C without shaking. At various times blood films were made and either stained with Giemsa or treated by the acid-elution method[11–13] modified to demonstrate Hb F-containing cells while at the same time allowing adequate morphological characterisation of parasites.

P. falciparum preferentially invades young, metabolically active red cells[14]. As this study was designed to compare the rates of invasion and growth *in vitro* in cells containing Hb A with those containing Hb F, and as these cells might be of different metabolic ages, a preliminary series of ments was carried out to examine the effect of cell the rates of invasion and growth of *P. falciparum in vitro* system. Normal red cells were fraction age[15,16] and each fraction was mixed separately fected cells. In each case the rate of invasion of the red cell population was significantly greater than tha older cells (Table 1). The further growth and devel of the parasites in the different aged cell populatio assessed both by morphological examination (Table ^{3}H-isoleucine incorporation[17,18]. Parasite growth by either method was similar in each cell fraction. this *in vitro* system the parasites preferentially young metabolically active cells but there is virtu difference in the rates of development in red cells o ing ages.

To determine whether Hb F has any eff invasion of red cells by *P. falciparum*, the rates of invasion of Hb A and Hb F-containir were compared. Hb A-containing cells were from normal adults. Hb F-containing cells derived from four sources—normal newborn infants aged 3–6 months, homozygotes for the British hereditary persistence of foetal haemoglobin (HPFI heterozygotes for the African form of HPFH[20]. Th of these Hb F-containing cells was based on the f rationale. The red cells of newborn infants contain Hb F and are metabolically younger than those of

Table 1 Invasion and development of *P. falciparum* in no cells of different ages

Cell fraction	Invasion: Reticulocytes (%)	Invasion: Parasites (%)	Development: Developmental stages of 1 sites per cell fractio R S	R L	T S	T L	S S	S L
Whole blood	0.4	5.0	0	3	40	30	11	1
Top	2.5	7.9	0	2	39	32	11	2
Middle	0.6	3.6	0	4	36	29	14	3
Bottom	0.2	3.3	0	3	36	24	16	1
Whole blood	2.4	5.1	0	1	31	37	14	1
Top	9.6	8.9	0	3	40	26	10	2
Middle	1.5	4.5	0	2	42	24	10	2
Bottom	0.1	3.2	0	0	49	21	10	1

Uninfected adult red cells were centrifuged at 2,500*g* for 1 h Fractions of approximately 0.2 ml were removed from middle and bottom of the column of red cells and from who Reticulocyte counts were done to confirm separation on the age. Suspensions of schizont-infected cells were added to ea tion and the mixtures were incubated as described in the t percentage of ring forms in each fraction arising from a fi culum of schizonts is shown for two experiments. At least 1 cells were examined from each fraction. In each case a χ^2 test a significantly greater percentage of parasites in the top (yo as compared with the bottom fraction ($P < 0.01$). T parasite development smears were made after 53 h in culture maturity of parasites in 100 singly infected cells in each was assessed. Rings (R) were forms without pigment, trop (T) had a single clump of pigment and a single nucleus, schi had more than one nucleus, gametocytes (G) had diffuse and abnormal forms (A) were those in which there was no cytoplasmic differentiation on staining and which were p The rings, trophozoites and schizonts were further subdivi small (S) and large (L) forms. There was no significant d in the pattern of development of the parasites between the cell fractions.

adults[21]; by making an artificial mixture of newborn-infant and adult cells it would be possible to compare the rates of invasion of two red cell populations of different metabolic ages with Hb F in the 'younger' cells. In the blood of infants aged 3–6 months Hb F production has almost ceased and the newly-produced red cells contain almost entirely Hb A; in the same blood sample it would be possible to examine the relative rates of invasion of Hb A and Hb F-containing cells, in this case the latter being the metabolically older population. The red cells of homozygotes for the British form of HPFH contain increased amounts of Hb F which is unevenly distributed, some cells carrying mainly Hb F and others mainly Hb A[18]. Since the metabolic ages of these populations are similar it would be possible to study the relative invasion rates of Hb F and Hb A-containing cell populations of the same metabolic age in the same blood sample. The red cells in the African form of HPFH, apart from their increased levels of Hb F, are similar in every way to those of normal adults[19]. The results are summarised in Table 2. In the mixture of newborn and adult red cells the Hb F-containing cells were preferentially invaded, in the red cells of the 3–6-month old infants there were significantly more parasites in the Hb A-containing cells, and in the red cells of the British HPFH homozygotes the Hb F and Hb A-containing populations were equally invaded. The rates of invasion of the African HPFH cells were identical to those of normal adult cells in parallel cultures. These experiments show that Hb F has no direct effect on the rate of invasion of red cells by *P. falciparum* and provide further evidence that invasion is a cell-age related process.

A further series of experiments was carried out to compare the development of *P. falciparum* in Hb F-containing cells with that in cells which contain mainly Hb A. In addition to the cells used in the experiments described above a mixture of normal adult cells and cells from a compound $\beta^0/\delta\beta$ thalassaemia heterzygote which contain almost 100% Hb F[22] were studied. The cells were infected

Table 2 Invasion by *P. falciparum* of red cells containing varying amounts of foetal haemoglobin

Cell type	Source of Hb F-containing cells: Adult+cord Parasites (%)	F/A	British HPFH Parasites (%)	F/A	African HPFH Parasites (%)	F/A	Infant Parasites (%)	F/A
A	5.2		10.8		3.1		7.4	
I	—	2.3	8.9	0.9	—	1.0	2.9	0.3
F	12.1		10.1		3.0		2.4	
A	5.1		9.6		7.8		4.3	
I	—	2.6	9.0	1.1	—	0.9	1.6	0.2
F	13.2		10.2		6.7		0.8	
A	5.5		4.3		6.2		12.2	
I	—	1.8	4.9	1.1	—	1.0	7.0	0.5
F	9.9		4.9		6.1		5.8	

Experimental conditions as described in the text. Where Hb A and Hb F-containing cells were present in the same culture, smears were stained by a modification of the acid elution technique to distinguish between the following groups of cells:— those containing predominantly Hb A where the haemoglobin was completely eluted (A), cells containing Hbs A and F (intermediate or I cells) and cells containing mainly Hb F (F cells)[13]. Since the mixture of adult and cord cells was artificial the group of I cells has been omitted. The African HPFH and adult control cells were grown in parallel and not in the same wells, as the acid elution technique does not readily distinguish African HPFH cells from those containing Hb A in an artificial mixture. In these cases the intermediate (I) group is omitted. At least 2,000 cells were counted in each experiment. The difference in parasitisation of the A and F cells in the adult/cord and infant cell experiments was significant at the 1% level. There was no significant difference between the parasite counts in the A and F populations in the British or African HPFH red cells.

Table 3 Development of *P. falciparum* in cells containing varying amounts of foetal haemoglobin

Blood type	Cell type	Developmental stages of 100 parasites per group: R S	R L	T S	T L	S S	S L	G	A
Cord	A	0	0	25	58	8	5	2	2
	F	0	3	64	23	3	0	2	5
	A	0	1	29	48	17	2	0	3
	F	0	3	78	14	0	0	0	5
$\beta^0/\delta\beta$ Thalassaemia	A	8	10	44	28	6	0	0	4
	F	34	29	25	9	0	0	0	3
	A	10	30	25	27	7	0	0	1
	F	42	38	17	1	0	0	0	2
British HPFH	A	3	21	31	34	8	0	1	2
	I	14	26	28	20	6	0	0	6
	F	26	10	27	18	1	0	0	18
	A	21	5	4	8	30	19	2	1
	I	45	14	4	11	20	3	0	3
	F	46	5	8	17	13	7	0	4
African HPFH	A	1	6	27	20	19	9	0	18
	F	1	10	33	20	9	3	0	24
	A	0	3	45	34	7	1	0	10
	F	0	4	54	24	1	0	1	16
Infant	A	21	43	14	9	11	3	0	0
	I	22	68	8	0	2	0	0	0
	F	24	67	7	2	0	0	0	0
	A	10	31	33	12	9	0	0	5
	I	30	30	28	8	0	0	0	4
	F	28	34	28	4	0	0	0	6

Experimental methods are described in the text and the designation of cell type and the development stages of the parasites are the same as those in Table 1. All cells were cultured in the same wells except for the African HPFH and Hb A-containing control cells which were in parallel. There is a significant retardation of development of parasites in the Hb F-containing cells in each case.

in vitro and at various times smears were made from the cultures and a comparison of the maturity of parasites in the Hb A and Hb F-containing cells was made. The results of representative experiments are summarised in Table 3. There was a significant reduction in the number of mature forms of *P. falciparum* in the Hb F-containing cells regardless of their source or age. Hence the presence of Hb F in red cells causes retardation of growth of *P. falciparum in vitro*. Since it was observed in red cells containing Hb F obtained from both infants and adults with several different genetic conditions, this phenomenon is probably related to the presence of Hb F and not to other metabolic properties of the red cells. Further evidence that this is the case was obtained in those experiments in which it was possible to define red cells which contained intermediate amounts of Hb F (Table 3). The degree of retardation of growth of *P. falciparum* in these cells was approximately intermediate between that in cells which contained predominantly Hb A or Hb F suggesting that the degree of retardation of growth of the parasite is broadly correlated with the level of Hb F in the red cell. Since the phenomenon was observed in the red cells of the African HPFH heterozygote, it seems likely that growth retardation can result from as little as 7 pg Hb F per cell.

Whether Hb F causes relative retardation of growth and development of *P. falciparum* by its high oxygen affinity, different primary structure from that of Hb A, or by another mechanism, remains to be determined. Whatever the mechanism this phenomenon, taken together with the retardation of the rate of decline of Hb F production over

the first year of life in infants heterozygous for β thalassaemia, offers a possible mechanism for the maintenance of the β thalassaemia polymorphism. Any property of the red cell which causes even minor growth retardation or interferes with synchronisation of development of the parasite might provide some degree of protection, particularly at a stage of development when passive immunity is waning and active immunity is not established. Further data on the levels of Hb F which are required for protection, and on the intracellular levels of Hb F in such heterozygous infants during their first year is required to test this hypothesis. Its particular attraction is that because the rate of decline of Hb F is retarded in infants with β chain structural haemoglobin variants as well as those with β thalassaemia[6], it provides a unifying mechanism for the high gene frequencies of the β-chain haemoglobinopathies. It does not, of course, exclude the operation of other protective mechanisms such as have been proposed for cells containing Hb S[1–4].

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SHORT COMMUNICATIONS

Separation of viable schizont-infected red cells of *Plasmodium falciparum* from human blood

A simple method of preparing high concentrations of red blood cells containing viable *P. falciparum* schizonts is described. In this procedure cellulose powder is utilized to free infected blood of white cells (Homewood and Neame, 1976) and schizonts are concentrated by fractionation in Plasmagel as used by Coulson and Chalmers (1964) to concentrate lymphocytes. This method has been of value in studies involving the *in vitro* culture of *P. falciparum* and may be of use in preparing antigen or merozoites.

Concentration of schizonts was carried out under sterile conditions as follows. Blood, heavily infected with late, synchronous *P. falciparum* rings, was obtained by venepuncture from Gambian children and collected into heparin (10 iu/ml). Removal of white cells was achieved by absorption with Whatman CF11 cellulose powder. Five ml of the infected blood was layered onto a column containing 20 cm^3 of cellulose powder pre-wetted with phosphate-buffered saline and packed into a 50-ml glass syringe plugged with non-absorbent cotton wool. The red cells were washed into a two-litre flask with 100 ml of supplemented T.C. Medium 199 (Phillips *et al.*, 1972). The flask was gassed with 5% CO_2/95% air, stoppered and incubated at 37°C with gentle shaking. The red cells emerging from the cellulose columns were largely free of white cells and showed no decrease in percentage parasitaemia.

The parasites were usually grown *in vitro* to the late schizont stage taking on average about 24 hours. However, in the cases documented a few shizonts had ruptured, giving rise to a new generation of rings. This enabled the effects of Plasmagel on the ring form to be observed.

The red cells were separated from the culture medium by centrifugation at 600 **g** for five minutes and were reconstituted to a haematocrit of 40% with medium 199 in a 15-ml centrifuge tube. To this red cell suspension was added various volumes of Plasmagel (3% w/v solution of gelatin in physiological saline—Laboratoire Roger Bellon, Neuilly) at 37°C and gently mixed. After sedimentation of the cells at 37°C, two distinct phases were visible with concentration of schizont-infected cells in the upper layer. The highest concentration of schizonts was obtained at a red cell suspension to Plasmagel ratio of 2 : 1 (Table 1). At this ratio the concentration of parasites increased with increasing time of sedimentation up to 15 minutes; thereafter the gain was minimal (Table 2). If left long enough 100% parasitized cells could be obtained but this may be at the expense of the viability and

TABLE 1

The effect of red cell suspension to plasmagel ratio on the concentration of schizont-infected red cells using a sedimentation time of ten minutes

Red cell suspension: plasmagel ratio	*Total red cells counted*	*Number of schizonts*	*Number of rings*	*Percentage of schizonts*	*Percentage of rings*	*Enrichment* (% *schizonts post-concentration*) / (% *schizonts pre-concentration*)
1 : 0	613	24	16	3·9	2·6	0·7
2 : 1	508	418	0	82·3	0	15·2
1 : 1	629	369	3	58·7	0·5	10·9
1 : 2	547	123	11	22·5	2·0	4·2

TABLE 2

The concentration of red cells containing P. falciparum *schizonts with increasing time of sedimentation*

Time (minutes)	*Cells*	*Schizonts*	*Rings*	*% schizonts*	*% rings*	*Enrichment*
Pre-conc.	637	27	32	4·2	5·2	—
5	581	212	16	36·5	2·8	8·7
10	533	348	5	65·3	0·9	15·5
15	557	427	7	77·0	1·3	18·0
20	540	418	2	77·4	0·4	18·4
120	100	100	0	100·0	0·0	23·8

number of parasites recovered. The results in both tables indicate that ring forms (from the next generation of parasites) are not concentrated by this method, presumably because they do not markedly alter cell buoyancy.

The method is simple, rapid, repeatedly produces good concentrations (80–90%) of schizonts and uses minimal physical manipulation (e.g. washing cells at any stage of the procedure was found to be unnecessary). In these respects it has advantages over existing methods (Williamson and Cover, 1975). Furthermore, parasites thus obtained were consistently found to be capable of invading and developing in fresh red blood cells.

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