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BLOOD GROUP POLYMORPHISMS

IN SOUTHERN AFRICA

AND INNATE RESISTANCE TO

Plasmodium falciparum.

Application of Flow Cytometry to the evaluation of Malaria parasites in continuous in vitro Culture

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Stephen Paul Field.

A research report submitted to the Faculty of Medicine, University of the Witwatersrand, Johannesburg, in part fulfilment of the requirements for the degree of Master of Medicine (in the branch of Haematology)

Johannesburg 1992.

Ethics approval was obtained from the

Committee for Research on Human Subjects (Medical),

University of the Witwatersrand.

Clearance Certificate Protocol Number 02/03/91.

Abstract

The observation by Haldane in 1949 that the distribution of malaria and certain thalassaemias were similar and that the former disease must be a selective force for the continued existence of the latter by preservation of the heterozygotes. This theory which later became known as "the malaria hypothesis" has been applied to other inherited conditions such as G6PD deficiency, membranopathies, certain blood group polymorphisms, other heamoglobinopathies such as sickle cell disease, blood group polymorphisms and more recently HLA phenotypes.

It has been shown that the Duffy blood group antigens are the receptors for *Plasmodium vivax* and since these antigens are lacking in most black Africans this species of malaria is virtually absent in Africa. It has also been shown that the glycophorins are at least in part the receptors for *P.falciparum*. Several variants of the glycophorins exist and the biochemistry and, where known, the molecular mechanisms by which these arise is reviewed. Experimental work is carried out to establish the growth characteristics of *P.falciparum* in an *in vitro* culture system using cells with glycophorin variants on their membranes. Three such variants were compared to normal cells and two (S-s-U- and Dantu) were found to be partially resistant to invasion by *P.falciparum* merozoites whereas the third (Henshaw) was found to be no different to controls.

A flow cytometric technique using the dye Thiazole Orange was adapted for use on the Epics® Profile II flow cytometer to enumerate the parasites. This

technique was compared to microscopy and tested for reproducibility and the results are presented. The ability of the merozoites to invade red cells was also assayed by [H^3] hypoxanthine incorporation.

In addition to being partially resistant to invasion Dantu cells appear to impair the normal division of the trophozoite stage and the resultant schizont has fewer merozoites than normal controls.

Declaration

I declare that this research report is my own, unaided work. It is being submitted for the degree of Master of Medicine (in the branch of Haematology) in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

Stephen Paul Field

16th April ,1992

Dedication

This report is dedicated to my ever patient wife, Wendy, and my three children David, Kevin and Barry.

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1 INTRODUCTION

Malaria has been and still is a significant cause of mortality and morbidity in man. Garnham (1966) believes that this parasitic infection is "the greatest single killer of the human race" . It is estimated that, despite global efforts to eradicate the disease over the last 40 years, in 1990 there are 270 million new cases of malaria each year and that 2.1 billion people live in areas with malarial transmission with at least one million deaths annually (Hoffman and Martinez 1990). In Africa south of the Sahara it is estimated that some 90 million cases of clinical malaria occur per year and the parasite carrier prevalence may be of the order of 250 million (WHO 1990). In the endemic areas children, whose immunity against the disease has not yet developed, are the main victims with the highest toll being in those aged between 6 months, when passive immunity declines, and 4 years at which time acquired immunity has developed (Haworth 1988).

1.1 Life Cycle of the Malaria Parasite.

Malaria parasites are inoculated into man in the form of sporozoites when the infected female *Anopheles* mosquito takes a blood meal.The sporozoites migrate from the bite site via the blood stream to the liver where they undergo the primary exo-erythrocytic cycle. This cycle occurs in the hepatocytes which culminates with the rupture of the infected cells (liver schizogony) and release of merozoites into the liver sinusoids. Here they invade red cells thus commencing the erythrocytic cycle. The period over

which the exo-erythrocytic cycle extends is characteristic for each species and is known as the prepatent phase (see Table I). The patient is asymptomatic during this phase only developing clinical illness some time later when the parasitaemia reaches a critical level. This interval between infection and the appearance of clinical signs and symptoms is known as the incubation period.

	Pfalciparum	*Pmalariae*	*Pvivax*	*Povale*
Common Name	Malignant Tertian	Quartan	Benign Tertian	Ovale Tertian
Prepatent period (days)	5.5	15	8	9
Incubation period (days)	15	17	28	12
Erythrocytic cycle (hours)	48	72	48	50
Hypnozoites	No	No	Yes	Yes

Table I Characteristics of the human infecting malaria species.

P.vivax and *P.ovale* differ from the other two human infecting species in that the sporozoites injected by the mosquito , in addition to the normal primary hepatic cycle, form latent forms known as hypnozoites. These hypnozoites remain in the hepatocytes for a long period, from months to several years, and are responsible for the relapses characteristic of the benign tertian malarias. The merozoites, once released into the liver sinusoids, invade red cells and thus initiate the erythrocytic cycle. Within the red cell the parasites grow rapidly and a vacuole forms in the cytoplasm giving the appearance of the characteristic ring forms. Growth continues, the vacuole becomes less distinct, pigment formation takes place, and the parasite takes on the more

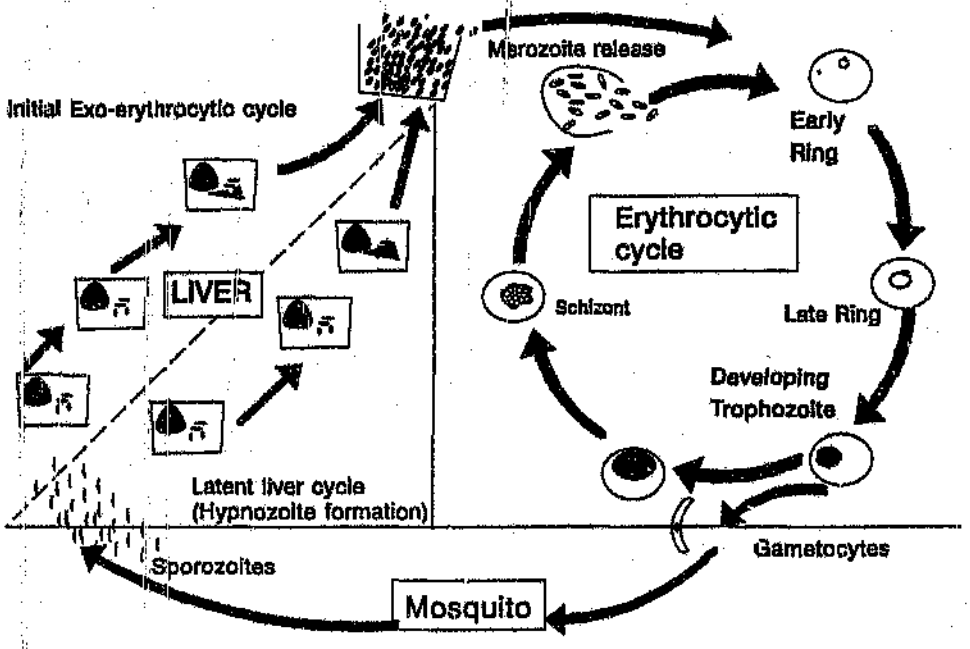


Figure 1 The life cycle of the malaria parasites. Note that only *P.vivax* and *P.ovale* produce hypnozoites in the liver.

globular appearance of the maturing trophozoite. Schizonts are formed when the nucleus begins to divide into several discrete nuclei (hence the name given to the species *Plasmodium*- many nuclei within one plasma). Segmentation then takes place with division of the cytoplasm and the formation of merozoites. The host cell lyses and releases the merozoites into the blood stream where they are can invade other red cells and commence this asexual cycle again. Under certain poorly understood conditions, such as an adverse environment within the host, the trophozoites do not undergo schizogony but develop into micro or macro gametocytes which, if taken up by an *Anopheline* mosquito, undergo sporogony in the insect vector and thus can initiate another cycle.

1.2 Origins of Malaria.

Malaria has been known throughout recorded history with reports that resemble the disease emanating from the ancient literature from China, Egypt, Greece, Italy and India although it is not always easy to readily identify the diseases documented in the historical records (Laderman 1975, Nurse 1986). Indeed Bruce-Chwatt and de Zulueta (1980) believe that malaria "must have afflicted man since his earliest days" and probably affected man's hominid forebears. The consensus in much of the literature, although speculative, is that the plasmodia probably originated in Africa. This occurred during the Eocene epoch of the tertiary age (approximately 50 million years ago) with the early human forms developing with man and the upper apes during the Pliocene and Pleistocene (1 to 2.5 million years ago). The parasite spread out of Africa relatively recently, with the transition of human behaviours from the hunter-gatherer to the agricultural-settler culture, via the upper Nile valley to Europe, India and China (Bruce-Chwatt 1965). A second focus of plasmodial evolution may have occurred in Southeast Asia (Sergiev and Tiburskaya 1965) over the same time span.

It has long been thought that the hallmark of a successful parasite is one that is able to obtain all its requirements from its host without adversely changing the lifespan of that host (Bruce-Chwatt 1965, Laderman 1975). It is for this reason that *P.falciparum* is thought to be the most recently evolved of the human infecting species in that it is the most lethal whereas the other species have reached an equilibrium with their hosts in that they cause an illness,

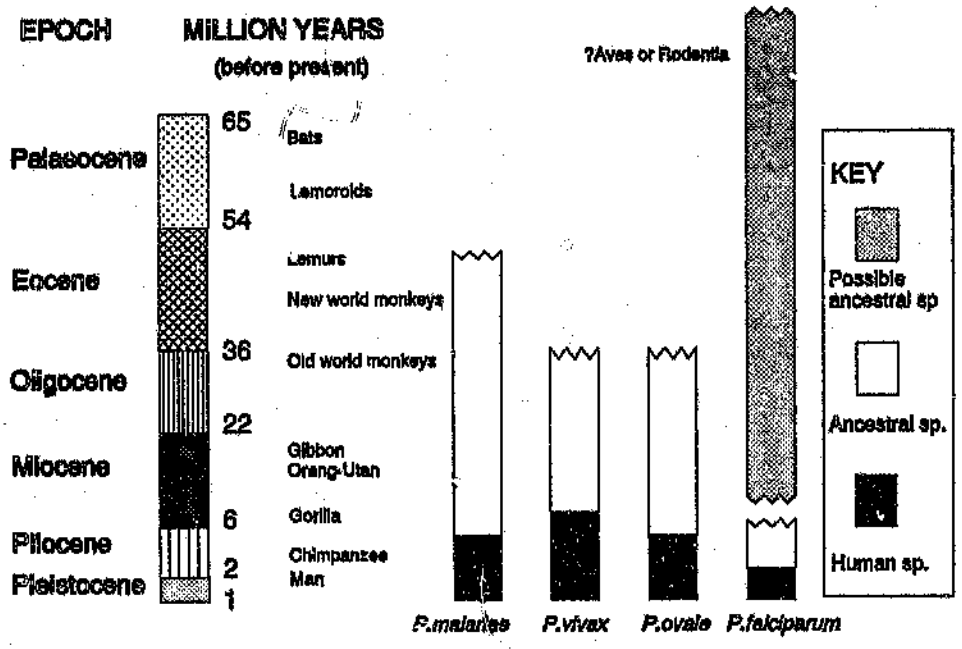


Figure 2 The hypothetical time scale of the evolution of the malaria species

which although unpleasant, is very rarely fatal. The hypothesis of greater virulence indicating recent origins has, however, been challenged (Garnham 1988) on the grounds that *P.falciparum* showed greater resemblance to avian and rodent malarias rather than to the primate malarias. The argument against the theory that *P.falciparum* is the youngest of the human infecting species assumes that the evolution of the parasites parallels that of its host. The evidence that *P.falciparum* is phylogenetically closer to the avian and rodent species is based firstly on the morphology of sporogonic stages and secondly on studies of the composition of parasite DNA. Sinden *et al* (1978) found that the ultrastructure of *P.falciparum* macrogametocytes in meiosis formed an intranuclear spindle with a single pole such as is found in avian and rodent species and is unlike that of primate malaria. McCutchan *et al* (1984) analyzed the purified DNA samples of *P.falciparum, P.knowlesi* (a simian malaria) and *P.berghei* (a rodent malaria) for their deoxyguanosine·deoxycytidine (dG·dC) content by determination of the melting temperature (T_M). It was found that *P.falciparum* and *P.berghei* had an dG·dC content of 18% whereas the *P.knowlesi* dG·dC content was 30%. Caesium Chloride (CsCl) centrifugation gradient studies confirmed these results and showed that the avian species *P.lophurae* had similar dG·dC content to *P.falciparum* . The monkey malaria species *P.fragile* and *P.cynomolgi* along with *P.vivax* all showed dG·dC contents of 30% although the latter two species had minor populations of 18% dG·dC content which Garnham (1988) believes may represent hypnozoites. This is difficult to accept since these forms are found only in the liver cells and therefore would not be recovered from peripheral blood. Hybridization studies with various

probes again showed that *P.falciparum* was closer to the avian and rodent malaria species. There is also a school of thought that the morphology, life cycle and disease produced are so unlike the other three human malarias that *P.falciparum* should be placed in its own genus *Laverania* as it was designated shortly after its discovery by Alphonse Laveran. The consensus is that this species belongs within the genus *Plasmodium* and subgenus *Laverania* (ie *Plasmodium (Laverania) falciparum*).

P.malariae, which produces benign quartan malaria in man and chimpanzees but not other apes or monkeys, is thought to be the phylogenetically oldest human species. Garnham (1966) postulates that the quartan malarias originally evolved in the lemurs and lower monkeys with the higher ape and human species appearing later. This has resulted in a parasite that is characterised by its slow development in both primate and insect hosts and the persistence, with periods of latency, of the infection in man for many years. The mechanism of latency displayed by this parasite is poorly understood since no evidence of any late relapse forms of the parasite (hypnozoites) could be recovered from livers of chimpanzees after inoculation with infected blood or injection of sporozoites (Bruce Chwatt 1985). The re-emergence of clinical infection after a period of latency is thought to be a recrudescence of the erythrocytic forms which had persisted, in small numbers, from the primary infection. This species also has a patchy distribution throughout the world with the greatest density being in the least developed parts of Africa, India and SE Asia. A possible reason for this is that the parasite is part of a zoonosis with the higher apes and as these animals are endangered the

survival of the parasite is similarly affected. Knowles *et al* (1930) suggested that *P.malariae* is a senescent species of ancient origin and is now in the course of disappearance.

The ancestral versions of *P.vivax* and *P.ovale*, the tertiary malarias, probably appeared in the oligocene epoch 35 million years ago in the old world monkeys. These species are very similar and probably evolved along a common pathway diverging only in recent times. The major difference between the two species is the dependency of *P.vivax* on the presence of the Duffy blood group antigens on the red cell membranes which are essential for merozoite penetration of the cell whereas *P.ovale* merozoites are able to invade the red cell independently of these antigen determinants. *P.ovale* is probably an adapted form of *P.vivax* as there are human populations in Africa who have very low gene frequencies for the Duffy antigens Fy^a and Fy^b. The production of hypnozoites in the benign tertian Plasmodia which allows the parasite to survive in a single host for many years with only periodic relapses of the illness can be seen as a survival mechanism. This is an indicator that the host-parasite relationship has reached equilibrium in phylogenetic terms. The latent periods also enable these species to survive the seasonality of its insect vector in the colder climatic regions in which they are found (for example the Russian sub-species *P.vivax hibernans*).

It is interesting that the simian species, *P.knowlesi*, which occasionally infects man, is also dependent on the Duffy antigens. This species, however, produces an illness similar to that caused by *P.falciparum* with high parasitaemias and

fulminant disease. Moreover the ring forms produced by *P.knowlesi* are morphologically comparable to those produced by *P.falciparum* and the late forms show the same tendency to disappear from the peripheral blood to undergo schizogony in the internal vessels (Garnham 1988). Unlike in the benign tertian species, hypnozoite production has not been demonstrated in *P.knowlesi*. It can be argued that *P.knowlesi* probably evolved independently of the benign tertian species and phylogenetically is a much younger species.

1.3 Adaptation of Man to Malaria

The long struggle between man and the malaria parasite has resulted in the development of several host defence mechanisms and the selection of genes which confer some degree of protection against the infection. It was Haldane (1949) who first recognised that high frequencies of the thalassaemias and other haemoglobinopathies occurred in the endemic malarial areas. He proposed that these high frequencies resulted because these genetic disorders, in the heterozygote state, may confer some protection against malaria. This theory has become known as "the malaria hypothesis" and has later been shown to apply to blood group polymorphisms, Glucose 6 Phosphate dehydrogenase (G6PD) deficiency and some red cell membrane disorders as well as the haemoglobinopathies. More recently HLA polymorphisms (both class I and II) were added to the list (Hill *et al* 1991). This review of the literature will focus on the role afforded by the blood group antigens as protective mechanisms against malaria. The role of other factors is adequately reviewed elsewhere (Nagel & Roth 1989, Nagel 1990, Weatherall 1987), and this is not directly relevant to this report.

1.3.1 The Blood Group Antigens

Certain of the blood group antigens have been shown to participate in the process by which the merozoite of various species invades the erythrocyte. These have been extensively studied by examining the ability of the parasite to invade red cells with (or lacking) certain blood group antigens and their variants. Most of the work has been done since Trager and Jensen (1976) successfully developed their method of *in vitro* cultivation of malaria parasites. These studies, which brought to light the importance of the Duffy and glycophorin associated blood groups are reviewed below. Epidemiological studies to establish an association between blood group polymorphisms and malaria are surprisingly scarce. Mourant *et al* (1976) have collated data from many sources and produced tables of blood group frequencies which enable predictions of likely antigens that may play some part in the protection of populations against malaria.

1.3.1.1 The Duffy Blood Group System.

Attention was drawn to the role of the Duffy blood group system and its relationship to plasmodial infection when Miller *et al* (1975) linked the earlier observation that a high percentage of African and American blacks are resistant to *P. vivax* (Boyd and Stratman-Thomas 1933) and that the majority of these populations lacked the Duffy blood group antigens Fy^a and Fy^b (the *FyFy* genotype). These findings were confirmed by Miller *et al* (1976) by feeding P.vivax infected mosquitoes on human black and white volunteers. All the white individuals, who were presumed to have either Fy^a or Fy^b red cell antigens, and Duffy positive black volunteers were all infected whereas

the black individuals with the *FyFy* genotype were resistant to the parasite. Since it is not possible at present to culture *P.vivax* the hypothesis was tested using the simian malaria parasite *P.knowlesi* which is able to invade human red cells and can be successfully cultured using the method of Trager and Jensen (1976). Duffy negative cells were found to be resistant to this parasite. It was also found that treatment with chymotrypsin which removes the Duffy blood group determinants rendered the cells resistant to *P.knowlesi* (Miller *et al* 1975, Mason *et al* 1977). Furthermore Mason *et al* (1977) demonstrated that erythrocytes from the great apes and the old world monkeys which all typed as Fy (a-b+) were all susceptible to invasion with *P.knowlesi* merozoites whereas the red cells of new world monkeys and lesser primates were not. An interesting observation made by these workers was the ability of *P.knowlesi* merozoites to invade the cells of chimpanzees (*Pan troglodytes*) and the kra monkey (*Macaca fascicularis*) after removal of the Fy^b antigens with chymotrypsin which would indicate that these primate species have an alternative red cell surface receptor for the parasite.

Haynes *et al* (1988) and Wertheimer and Barnwell (1989) provided further evidence of the role of the Duffy antigens by the isolation of proteins from *P.knowlesi* and *P.vivax* respectively that show binding specificity for this antigen. The *P.vivax* Duffy associated protein (PvDAP-1) which is 135-140 kDa in size has immunological cross reactivity with the *P.knowlesi* derived protein (PkDAP-1). This work on these two species of malaria strongly suggests that the Duffy antigen is a receptor which facilitates the invasion of merozoites into the red cell. This, however, is not true for the other human

species of *Plasmodia* all of which are able to invade red cells independently of the Duffy groups on the cell membrane.

Chaudhuri *et al* (1989), using murine monoclonal anti Fy 6 and an immunoblotting technique, have characterised the Duffy antigen on red cell ghosts. They found that antigen-antibody complexes that had been solubilised with detergent and subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) yielded a complex pattern of bands. This indicates that the Duffy antigens are part of a multimeric protein complex. One fraction of this complex designated as pD protein carries the antigenic determinants and is an integral membrane protein of 36-46 kDa which is not firmly associated with the cytoskeleton. It has the property of forming discrete oligomers of over 100 kDa. There are six other proteins which are associated with the pD fraction, four of which are present in all three Duffy antigen positive cell types (ie Fy(a+b-), Fy(a-b+), Fy(a+b+)). The other two associated proteins are only present in Fy(a+b-) and Fy(a+b+) respectively. None of the seven proteins have been identified in Fy(a-b-) cells.

The mechanisms of merozoite invasion are poorly understood as it appears that they attach to and deform the membrane of Duffy negative cells but are unable to undergo the second stage of invagination and interiorization of the erythrocyte (Dvorak *et al* 1975). Chaudhuri *et al* (1989) suggest that the parasite may require a multivalent association with the receptor which would be a complex of surface proteins composed of several subunits one set contributing to attachment and another to penetration.

1.3.1.2 The Glycophorins

The red cell receptors for *P.falciparum* merozoites are, at least in part, the integral membrane proteins known as the glycophorins. (Fasvol *et al* 1982 a,b; Facer 1983). Four glycophorins,designated as Glycophorins (GP) A to D, have been fully characterised at both the molecular and protein levels. More recently the gene for a putative fifth glycophorin (E) has been discovered and although the gene has been sequenced no protein products have been isolated. The GP E gene had previously been known as *inv.* The major properties of the glycophorins are summarised in Table II.

Property	GP A	GP B	GP C	GP D	GP E
Synonyms	SGP α	SGP δ	SGP β	SGP γ	
Major Blood Group antigens	MN Wr^b	SsU 'N' Henshaw	Gerbich:3	Gerbich:2,3	?
PAS positivity	85%	10%	4%	1%	?
Apparent Molecular mass (kDa)	36	20	32	23	?17
Copies/cell	500-900	300	50-100	20	?
Amino acids	131	72	128	107	59
O-linked sugars	15	11	12	6	?11
N-linked sugars	1	0	1	0	?

Table II The major properties of the glycophorins (Blanchard 1990, Kudo *et al* 1990, Vignal *et al* 1990)

Glycophorins A,B and E genes are all located on chromosome 4 and are arranged in tandem. The 3 genes are homologous in their 5' regions but variable in the 3' regions (Kudo *et al* 1990, Vignal *et al* 1990). Misalignment

of chromosome 4 during meiosis and unequal crossing over of chromatids may lead to gene deletions or hybrid genes. Some of these may be of significance with respect to the susceptibility to malaria of the erythrocytes which carry the aberrant gene products. The red cell expression of Glycophorin E has not yet been confirmed. The 5' cDNA sequence suggests that the N terminus should express the M or N blood group antigens but it may produce only a small number of copies per cell and no antigens are expressed. Alternatively the gene is silent (for example lacking an promoter sequence) and therefore there may not be any protein product.

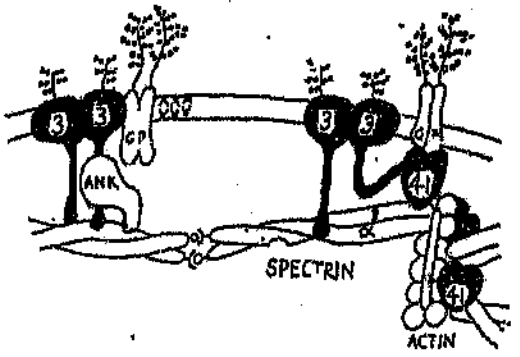


Figure 3 The components of the red cell membrane

The relationship the glycophorins have with other components of the red cell membrane is probably central to the process of merozoite invasion. These relationships are shown in Figure 3. The interaction of the C-termini of glycophorins A and C is with band 4.1, whereas the other three glycophorins have no such connection with the integral membrane proteins (Reid *et al* 1990). These authors suggest that the function of this relationship is to

regulate red cell shape. Biochemical analysis has shown that there is no homology between GP A and GP C (Blanchard 1990). The blood group antigens which are found on the glycophorins serve as useful markers and the absence or variance of one or more of these indicates that part or all of the glycophorin molecule is either missing or has been altered structurally (some of these are summarised in Table III.)

Red Cell type	Defect
En(a-)	GP A Absent
S-s-U- (S^U)	GP B Absent
Tn	Sialic acid and Galactose residues absent on alkali labile tetrasaccharide chains.
M^k	GP A and GP B absent
Cad	Modified alkali labile tetrasaccharide to a pentasaccharide.
M^s	Amino acid substitution position 4 N terminal GP A (Threonine → Asparagine)
He	Variant of N terminal of GP B (3 amino acids different from 'N' antigen)
Dantu	Hybrid GP A/GP B (δ-α)
Stones (St^a)	Hybrid GP A/GP B (δ-α)
Miltenberger Class V (MiV)	Hybrid GP A/GP B (α-δ)
Other Miltenberger antigens (Classes I-IV & VI-VIII.	Point mutations in either GP A or GP B. In some cases a hybrid (δ-α-δ)

Table III The variant glycophorins

1.3.1.2.1 Absent Glycophorin A En(a-)

Miller *et al* (1977) first suggested that the glycophorins play a role in the invasion of *P.falciparum* merozoites when they noted that the red cells of two individuals that lacked glycophorin A on their red cells (the En(a-) phenotype) showed relative resistance to infection by the parasite in culture. This was confirmed by Pasvol *et al* (1982 a,b) and Facer (1983) although the paleogenetic significance of this finding is unclear since this phenotype is rare. Two variants of En(a-) have been described (the Finnish and English types) each arising from separate molecular mechanisms and malaria is unlikely to be an agent influencing their selection.

1.3.1.2.2 Tn

Pasvol *et al* (1982) also found that the merozoites failed to invade cells from a donor that carried the Tn antigen. These cells have glycophorins that are deficient in sialic acid and galactose as the result of an acquired deficiency of ß-3-D-galactosyltransferase (Cartron *et al* 1978). This indicates that the sialic acid moiety of the glycophorins probably is the component required by the merozoite to facilitate the invasion process.

1.3.1.2.3 M^g

Cartron *et al* (1983) also found that M^gM^g cells are susceptible to merozoite invasion. These cells have antigens which differ from M or N as a result of an amino acid substitution at position 4 (threonine → asparagine). The

presence of an asparagine at position 4 results in a marked reduction of the glycosylation of the adjacent threonine residues at positions 2 and 3. This finding suggests that the glycosylated residues elsewhere on the glycophorin A molecule are adequate for invasion.

1.3.1.2.4 Cad

Cartron *et al* (1983) investigated a series of red cells that carried rare blood group antigens, all of which carried abnormalities of the sialotetrasaccharide chains of the glycophorins. These authors found that cells of the Cad type were resistant to invasion by *P.falciparum*. These cells have an additional sugar (N-acetyl-D-galactosamine) residue bound to the same galactose residue that carries the sialic acid (N-acetyl neuraminic acid) (Blanchard *et*

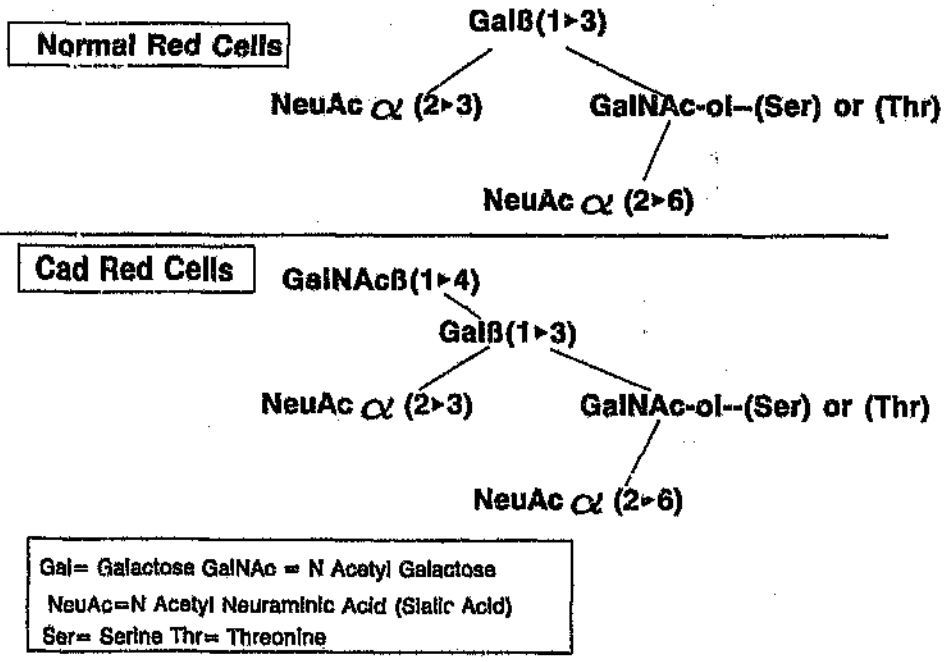


Figure 4 Configuration of the sugars on the alkali labile pentasaccharide on Cad erythrocytes.

al 1983). This additional sugar probably blocks merozoite attachment to the sialic acid.

1.3.1.2.5 Wr^b

One of the early putative malaria receptors was the antigen Wr^b which is an antigen found at the external membrane junction on glycophorin A between amino acid residues 55 and 70 (Ridgwell *et al* 1983). Pasvol *et al* (1982a) found that cells which lacked the antigen were less susceptible to invasion *in vitro* than normal cells. However his findings were not confirmed and other workers (Facer and Mitchell 1984) claimed contrary results indicating that this was not the red cell ligand for *P.falciparum* .

1.3.1.2.6 S-s-U- (S^U)

Pasvol (1982) and Facer (1983) found that cells of the S-s-U- type were significantly resistant *in vitro* to invasion by *P.falciparum* merozoites. This is a particularly important finding since the gene coding for the S-s-U- phenotype (S^U) has reached polymorphic frequencies in the malaria endemic Central and Equatorial Africa (gene frequencies for S^U range from 0.31-0.59 in pygmie populations in the Congo) (Lowe and Moores 1972, Jenkins and Ramsay 1986). The frequency of the gene is much lower in the relatively malaria free Southern Africa (for example gene frequency of 0.14 in Malawi, 0.86 in Zimbabwe and 0.00 in Natal South Africa)(Lowe and Moores 1972). Jenkins and Ramsay (1986) suggest that the advantages of the homozygous phenotype are such that the frequency of the S^U gene in the endemic regions should be higher but are kept down by a high incidence of fatal cases of

haemolytic disease of the newborn. Martin *et al* (1979) conducted a survey in Nigerian children presenting to Ibadan hospitals with malaria. Although numbers were small they found that the distribution of children with the S^U phenotype who had malaria was not significantly different from controls.

Recent work at the molecular level has shown that there are at least two different gene alterations that lead to this phenotype (Rahuel *et al* 1991) . The glycophorin B gene on chromosome 4 is composed of 5 exons (B1-B5). Southern blotting analysis, using the GPB-2 probe and a number of restriction enzymes, showed that the type I variant is a large deletion extending from exon B2 to B5. This finding concurs with that of Huang *et al* (1987). The whole glycophorin B gene structure is intact in the type II variant and the defect which gives rise to the expression of the S-s-U- phenotype is unknown but occurs at the transcription or translation level. It is not clear which of the two types is found in polymorphic proportions in Africa.

1.3.1.2.7 Enzyme Modified Red Cells

Several researchers have modified cells with enzymes (Miller et al 1977, Pasvol et al 1982a,b, Facer 1983, Breuer et al 1983). The terminal portion of glycophorin A is removed by trypsin (Issit 1985) and all studies demonstrated that removal of this membrane protein resulted in loss of the ability to invade red cells by the merozoites of *P.falciparum*. This not only confirms the En(a-) data but shows that the N terminal 39 amino acids are of importance to the process of invasion. Neuraminidase removes the sialic acid residues from the glycophorins which also renders the cells resistant to

invasion thus corroborating the observations made with Tn positive cells. Thus the role of the N-terminal portion of glycophorin A is probably the sugars they carry rather than the amino acid backbone *per se*. Chymotrypsin treated cells have no effect on the ability of *P.falciparum* merozoites to invade them but the invasion of *P.knowlesi* merozoites is blocked since this enzyme destroys the Duffy antigens.

1.3.1.2.8 Glycophorin binding proteins produced by merozoites

Camus and Hadley (1985) isolated a protein from supernatant fluids of cultured *P.falciparum* which binds to merozoites and red cells. This protein was found to have a molecular mass of 175 kDa and probably acts as a bridge between the erythrocyte and the invading merozoite. These workers also found that the protein produced is strain specific with respect to its merozoite binding properties but not its ability to bind to the glycophorins on the erythrocyte. Red cells that had been coated with the protein derived from one strain (Camps) blocked merozoites from another (FCR-3) and *vice versa*. This is illustrated in Figure 5.

The protein binds to the sialic acid moiety of the glycophorins providing further evidence of the role of these molecules as the merozoite receptor. The DNA encoding this protein (now called EBA-175) has been sequenced (Sim 1990) and its primary structure deduced. Synthetic fragments, based on predicted antigenic peptide sequences, were injected into rabbits and one such fraction, EBA-peptide 4, was able to raise an antibody which was inhibitory to *P.falciparum* merozoite invasion in culture. Unlike many other plasmodial

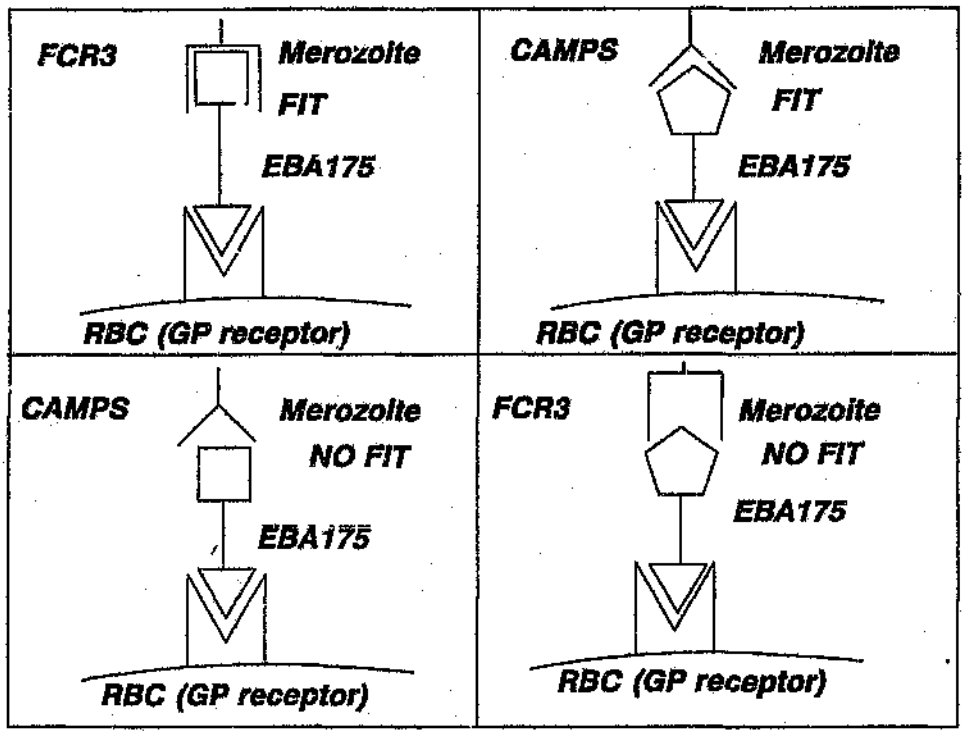


Figure 5 The strain specificity of the EBA-175. The GP binding is specific whereas the merozoite binding is strain specific.

antigens, which exhibit diversity within the species, the EBA-peptide 4 has been shown to be conserved at the nucleic acid level in at least six different strains of *P.falciparum* from many parts of the world (Orlandi *et al* 1990).

1.3.1.2.9 Receptor heterogeneity

All the work referred to above is strong evidence that the red cell receptor for *P.falciparum* is the sialic acid moiety of glycophorins A and B. There is, however, some evidence that it is not as simple as it may appear. Mitchell *et al* (1986) believe that there is some receptor heterogeneity among *P.falciparum* parasites. They base their argument on their observation that different strains of the parasite show variable abilities to invade Tn erythrocytes. One strain successfully maintained in the Tn erythrocytes (Thai-Tn) was compared to their parent strain (Thai-2) and the Camp strains of

P.falciparum . These workers demonstrated that the Thai-Tn parasites are able to invade neuraminidase treated normal and Tn erythrocytes whereas the other two strains showed very poor invasiveness to these cell types. An interesting facet of this work is the observation that Tn cells treated with neuraminidase were about 40% more susceptible to invasion by the Tn-Thai strain than untreated cells. They also showed that the Camp and Thai-2 strains were able to invade trypsinised cells better than the Thai-Tn parasites although this data should be interpreted with caution as the differences are small. Mitchell *et al* (1986) conclude from this work that there are two parasite membrane ligands, one sialic acid dependent and the other sialic acid independent and the variability between strains is due to either different numbers of the ligands or greater affinities for one or other of the red cell receptors. The argument for the presence of two red cell receptors is strengthened by the work of Hadley *et al* (1987) who showed that one strain of *P.falciparum* (7G8 strain) was able to invade red cells, albeit with only 50% efficiency, that lacked glycophorin A and B (M^kM^k cells). Dolan *et al* (1990), noting the findings of other workers (Mitchell *et al* 1986, Perkins and Holt 1988), investigated the invasion properties of 6 different strains of the parasite. They found that *P.falciparum* clones could be placed into two categories according to their ability to invade and grow in neuraminidase treated red cells. Type I parasites invade and grow in these cells although at a reduced rate as compared with untreated cells. Type II parasites invaded the treated cells very poorly with a sub group disappearing from culture (Type II-A parasites) . Type II-B parasites however are able to adapt to the conditions and although initially invasion rates are poor they survive in continuous

culture and invasion becomes more efficient. All parasite strains were subjected to Southern blot analyses, hybridizing with the recombinant pC4.H32 probe which detects interspersed repetitive elements, before and after each experiment. They demonstrated no difference in the unique hybridization patterns (DNA fingerprint) of type II-B parasites which shows that the parasites maintained their clonal integrity. The mechanism of adaptation is unknown but Dolan *et al* (1990) postulate that a gene switching mechanism may exist. This on the basis of the experimental work done is plausible but the necessity for such a mechanism *in vivo* is not clear since the aberrant glycophorins apart from S^U have not reached polymorphic proportions. In any case most of the type II-B strains occur outside of Africa and therefore the influence of S^U on the presence of the alternate gene is negligible.

1.3.1.2.10 Henshaw (He)

A variant of the terminal portion of glycophorin B which is present in polymorphic frequencies in Africa is phenotypically expressed as the blood group antigen Henshaw (He). This antigen appears to be unique to people of African origin.

Henshaw was discovered when an extra antibody was noted after rabbits had been immunised with human type M cells in the preparation of immune anti M anti sera (Ikin and Mourant 1951). These workers found that the antibody reacted with red cells of two Nigerians which typed as N with other antisera. They thought that they had rediscovered the antibody described by Landsteiner *et al* (1934) that had been evoked in rabbits by injection of the

cells of a West African by the name of Mr Hunter (hereafter the antigen became known by his name). Anti Henshaw was subsequently produced deliberately by the immunization of rabbits with the blood of a Nigerian laboratory technician Mr Henshaw (Chalmers *et al* 1953). This antibody was shown by Chalmers *et al* (1953) to be different from anti Hunter. Wiener and Rosenfield (1961), working again with immunised rabbits, found an antibody, which was designated anti M^e , that cross reacted with both M and He antigens independently. The rabbit which elicited the anti M^e had not been exposed to He positive cells which suggests that there is some homology between the antigenic structures of M and He. Subsequently, examples in human sera of anti He (MacDonald *et al* 1967) and anti M^e (McDougal and Jenkins 1981) were found.

Shapiro (1956) extensively investigated 7 South African black families and found that the Henshaw antigen segregated with particular M N S or s antigens in all but one case which typed as NNssHe unlike her siblings who all typed as MNssHe. The child's mother typed as MNssHe and the father NNss and in this family the He antigen was thought to be linked to M and s antigens. Shapiro (1956) postulated that the one case that failed to segregate as predicted either had a different father that with the NNssHe type of red cells or that there had been crossing over of the maternal chromosomes. The latter theory is probably correct as subsequent studies have shown that the Henshaw antigen is part of glycophorin B as are the S,s and U antigens but unlike M and N which are found on glycophorin A. They are all however coded by tandem genes on the long arm of chromosome 4

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(4q 28-31) (Cook *et al* 1980) and therefore are likely to segregate together unless crossing over had taken place.

Antigen	Amino Acid Sequence (N-terminal)	Glycophorin
M	ser-ser-thr-thr-gly * * *	A
N	leu-ser-thr-thr-glu * * *	A
'N'	leu-ser-thr-thr-glu * * *	B
He	trp-ser-thr-ser-gly * * *	B

* = glycosylation sites
ser= serine thr = threonine gly = glycine
leu= leucine glu = glutamic acid trp = tryptophan

Table IV The amino acid sequences for the blood group antigens located at the N-terminal of glycophorins A and B.

Judd *et al* (1983) studied cells of the rare genotype *MsHe/MS*^{U} and found that these cells were N quote ('N') negative. 'N' is the antigen which has the same amino acid sequence as N but is found at the N-terminal of glycophorin B (see Table IV) . Dahr *et al* (1984) sequenced tryptic glycopeptides from He heterozygotes and showed that the He structure represents an allelomorphic form of 'N' antigen thus concurring with the earlier findings of Judd *et al* 1983. The amino acid sequences of the various blood group antigens associated with the N-terminals of glycophorins A and B are shown in Table IV.

The He gene occurs throughout Africa and the gene frequencies are shown in Table V along with those for the Hu antigen. The gene frequency is

calculated by application of the Hardy-Weinberg equation and assumes that the He positive individuals include both heterozygotes and homozygotes.

The role that He plays in the innate resistance to malaria is not known. Since it does appear in polymorphic frequencies and is part of the glycophorin B molecule it is feasible to propose that this antigen may play some role in the resistance to *P.falciparum* merozoite invasion on the red cell.

The distribution of the polymorphism extending to outside the endemic areas is also argument against its role in malaria resistance. This however can be explained by the relatively recent migrations of people from the hyperendemic malaria areas to the virtual non malarial regions such as South Africa. There is great variation in the gene frequencies of He in the different indigenous peoples of Southern Africa. These are shown in Tables VI, VII and VIII which sub divide the populations on the basis of their language types.

It is interesting to note the variation within and between all the different groups of people. This may indicate that the populations with the higher frequencies for He have had greater exposure to the environmental factor influencing its selection. There is no discernable cline demonstrable and it is not obvious what this selective force may be, although malaria remains a strong candidate despite the fact that many of the people have migrated away from the endemic areas for this disease. The San people are still hunter gatherers who have avoided malaria as a result of their continuous migrations. However the areas in which they now live have become smaller as political

boundaries and agriculture have restricted the natural migrations of these people and therefore made them more susceptible to disease, particularly that confined to certain geographical regions.

Country	Henshaw (He)			Hunter (Hu)		
	n	% Pos	gene freq.	n	% Pos	gene freq.
Tunisia	244	0.8	0.0041			
Ethiopia	590	1.5	0.0077			
Gambia	124	3.2	0.0163	124	8.1	0.0412
Ghana & Nigeria	1428	2.7	0.0134	138	21.7	0.1153
Ghana	112	5.4	0.0272	29	31.0	0.1695
Ivory Coast	53	5.7	0.0287			
Nigeria	832	5.5	0.0280	349	10.9	0.0560
Tanzania	431	5.1	0.0259	93	2.2	0.0108
Uganda	563	3.6	0.0179	220	5.5	0.0277
Liberia	445	2.2	0.0113			
Niger	164	0.0	0.0000	164	0.0	0.0000
Somalia	233	0.0	0.0000			
Sudan	437	1.1	0.0057	337	3.3	0.0165
Upper Volta	23	0.0	0.0000			
C.African Republic	307	7.8	0.0399			
Lesotho	180	10.6	0.0542			
Botswana	428	17.1	0.0893			
Zambia	628	5.6	0.0283			
Zimbabwe	156	1.9	0.0097			
Namibia	389	6.2	0.0313			
S.Africa	4044	6.3	0.0322	30	26.6	

Table V The frequencies of the He and Hu genes in the indigenous peoples of Africa (Data from Mourant *et al* 1976)

	n	Positive	%	Gene frequency
G!aokx'ate	33	11	33.3%	0.1835
!Xõ	51	5	9.8%	0.0503
ǂhuã	36	2	5.6%	0.0282
G/wi	94	4	4.3%	0.0215
G//ana	50	3	6.0%	0.0305
Nharo	125	12	9.6%	0.0492
//au//en	111	22	19.8%	0.1046
!Kung, Dobe	258	49	19.0%	0.1000
!Kung, /ai/ai	65	5	7.7%	0.0392
!Kung, /du/da	64	3	4.7%	0.0237
!Kung, Tsumkwe	248	17	6.9%	0.0349
!Kung G!ãg!ai	36	3	8.3%	0.0426
Cumulative frequency	1171	136	11.6%	0.0599

Table VI Henshaw gene frequencies in the San people of Southern Africa. (Nurse & Jenkins 1977, Nurse *et al* 1985)

SA bantu speaking	n	Positive	%	Gene frequency
Nguni	123	5	4.1%	0.0205
Sotho/Tswana	764	57	7.5%	0.0380
Venda	105	4	3.8%	0.0192
Kavongo	349	18	5.2%	0.0261
Ambo	591	46	7.8%	0.0397
Herero	254	5	2.0%	0.0099
Cumulative frequency	2,186	135	6.2	0.0314

Table VII Henshaw gene frequencies in the Bantu speaking people of Southern Africa (Nurse & Jenkins 1977, Nurse *et al* 1985)

	n	Positive	%	Gene Frequency
Sarwa	79	5	6.3%	0.0322
Kwengo	36	0	0.0%	0.0000
Dama	79	0	0.0%	0.0000
Cumulative frequency	194	5	2.6%	0.0130

Table VIII Henshaw gene frequencies in the Khoisan speaking people of Southern Africa (Nurse & Jenkins 1977, Nurse *et al 1985)*

Whilst the migratory habits of this interesting group of people may be an argument against the hypothesis of Henshaw or any polymorphism within the group being selected on the basis of innate resistance to Malaria, the high frequency seen in some of the sub groups may well have occurred because of genetic drift in more recent times as a result of their restricted movement.

1.3.1.2.11 Hunter

The Hunter antigen was discovered by Landsteiner *et al (1934)* when the blood from a Mr Hunter injected into rabbits elicited an antibody other than the anti M that was intended by the exercise. The antigen that elicited this antibody appears to be polymorphic in Africa with the highest gene frequencies being found in West Africa (See Table V). Unfortunately anti Hu has only been elicited with the cells of Mr Hunter and some of his offspring and antisera are no longer available, consequently surveys in Southern Africa have been limited to very small numbers (8/30 in Johannesburg bantu speakers according to Shapiro 1956). The nature of the

antigen has not been determined and its role, if any, in protection against malaria is not known.

1.3.1.2.12 Dantu

Dantu is a low frequency red cell antigen which has to date been found mainly in people of negro origin (Contreras *et al* 1984). The first examples of the red cells carrying the Dantu phenotype were described by Contreras *et al* (1984) who characterised the antigen on the basis of its serological reactions. The antigen is named after a Mr Dantu, a partly negro man of South African origins, whose cells were found to react with the serum of a patient (Cam) which contained multiple antibodies. None of the antibodies that could be identified in Cam's serum corresponded to known antigens on Dantu's red cells and further testing in England and North America confirmed that the low frequency antigen on his cells was different to those already described. A second example of the antigen was discovered when a baby was delivered with a weakly positive Coomb's test and an eluate from the babies cells was found to react with the father's and Mr.Dantu's red cells. The father was later confirmed to be Dantu positive. Contreras *et al* (1984) describe several other propositi they have serologically characterised as Dantu including two which had been previously biochemically characterised by SDS PAGE and found to be non identical hybrid glycophorins. They had been designated as $(\delta\text{-}\alpha)^{Ph}$ (Tanner *et al* 1980) and $(\delta\text{-}\alpha)^{NE}$ (Unger *et al* 1981) thus establishing that the Dantu antigen existed as at least two biochemical variants. A third variant found in a caucasian subject (M.D.) was subsequently discovered and characterised (Pilkington *et al* 1985, Dahr *et al*

1989). The term (δ-α) indicates that the molecule is composed of the N-terminus of sialoglycoprotein δ (GP B) and the C-terminus of sialoglycoprotein α (GP A). The hybrid is thought to have resulted from misalignment of homologous chromosomes during meiosis and crossing over in an anti Lepore type arrangement. This is illustrated in Figure 6 which shows both the Lepore and anti Lepore types. The complimentary genetic configuration to Dantu (ie the (α-δ) Lepore type) has not yet been discovered although an (α-δ) hybrid, the Miltenberger class V (Mi.V) antigen, has been described in other population groups.

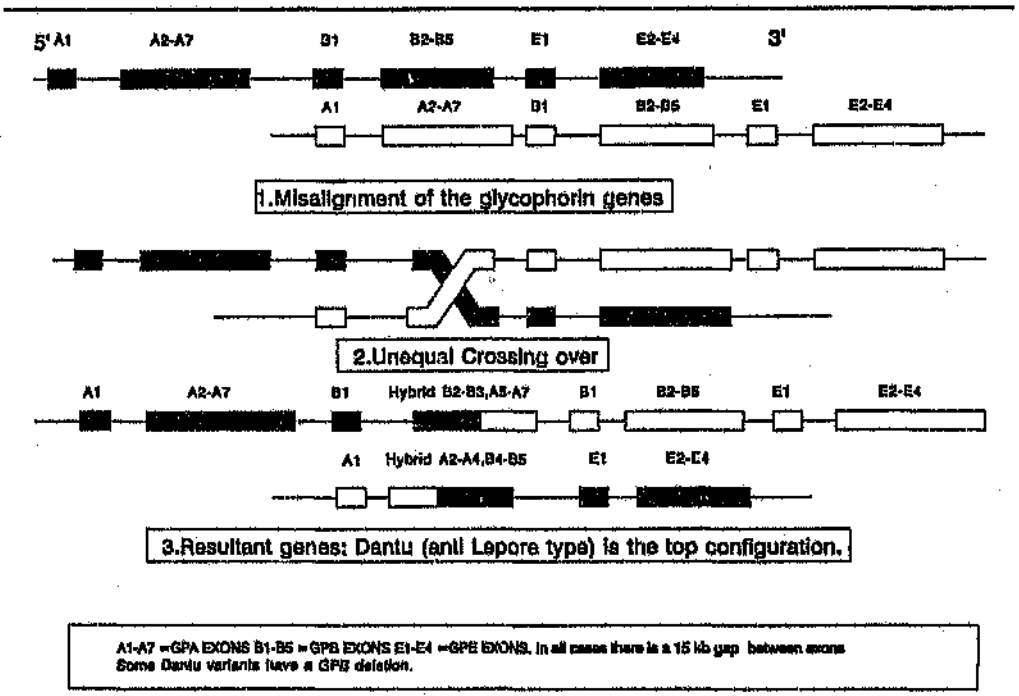


Figure 6 The molecular mechanism of the hybrid glycophorins (eg Dantu).

The Ph and NE Dantu variants react identically with anti Dantu but differ from each other with respect to the expression of normal glycophorins. The Ph type is characterised by the absence of normal GP B which suggests that

it is inherited in conjunction with the S^U gene (Tanner *et al* 1980). The family studied by Huang and Blumenfeld (1988) included several members with the Dantu phenotype which were also deficient in GP B. They showed that the entire coding sequence for GP B was deleted thus confirming the hypothesis of Tanner (1980). The red cells carrying the NE hybrid have a normal GP B but have reduced GP A (GP A:Hybrid GP ≈ 1:2,4) (Dahr *et al* 1987, Blumenfeld et al (1987). This is significant since in normal red cells the GP A is the most abundant of the glycophorins. The family studied by Huang and Blumenfeld (1988), the pedigree of which is further complicated by the presence of a second glycophorin variant (MiIII), appeared to have duplicated Dantu genes as well as a δ gene deletion (δ^0). Most Dantu cells have normal M expression using standard antisera which suggests the hybrid gene is linked to the αM gene (Huang and Blumenfeld 1988). All cases of Dantu however also express the N antigen but this is trypsin resistant which indicates that it is encoded at the N terminus of GP B (ie 'N'). Since the Ph variant has a deleted δ gene the N antigen detected must be part of the Dantu hybrid. The N detected in the NE variant must be mainly from the hybrid GP since 'N' on normal GP B is often undetectable by normal antisera. The expression of s in Dantu cells is of interest as the reaction with various antisera is variable. If the cell has a normal GP B present with the s gene present then the reaction with all anti s antisera would be positive as expected. In the absence of GP B where the only source of the antigen is on the hybrid, the reaction with the antisera may be weak (antigen is thus designated s^w) or with some antisera negative. The expression of U is dependent on the presence of the δ gene.

The influence of the Dantu antigen on the invasion of *P.falciparum* merozoites has not yet been determined. Dahr et al (1987), however, suggest that since the Dantu phenotype occurs much more frequently in blacks than in caucasians that it may confer a selective advantage against malaria. This hypothesis has not to date been tested. It is of interest to note that the Wr^b antigen is absent from the hybrid molecule (Blanchard *et al* 1987) although the original speculation that this antigen is a receptor for *P.falciparum* (Pasvol *et al* 1982a) has been challenged (Facer and Mitchell 1984).

The Dantu antigen is rare in Southern Africa and has not reached polymorphic proportions. Table IX shows the results of a large unpublished survey of various populations in the region (Moores 1992).

ORIGIN	NUMBER TESTED	POSITIVES
Durban	509	0
Cape Town	686	0
Port Elizabeth	303	1
Namibia	286	0
Zimbabwe	923	1
TOTAL	2707	2

Table IX Frequency of the Dantu phenotype in Southern African blacks tested with anti Dantu (Moores 1992).

1.3.1.2.13 Other Hybrid Glycophorins.

Other hybrid glycophorins of both the Lepore and anti Lepore types have been described. These express different antigens than the Dantu and the

junction points are different. The (δ-α) hybrid which expresses the Stones (St^a) antigen is composed of 99 amino acids comprising of the N-terminal 26-28 amino acids of GPB and residues 59/61-131 of GP A Blanchard *et al* (1987); Huang *et al* (1989). Huang *et al* (1989) also showed the gene exists as a single copy in the genome which is tightly linked to the αM and δS genes.

The junction point of St^a gene is shifted 12 codons upstream from that of the Dantu gene. The Lepore type (α-δ) hybrid known as the Miltenberger class V (MiV) has also been molecularly characterised and it would appear that this is the reciprocal configuration to the St^a gene (Huang and Blumenfeld 1991).

Pasvol *et al* (1982) using an undefined α-δ hybrid of about 56 amino acids demonstrated partial inhibition of invasion by *P.falciparum* merozoites. Treatment of these cells with trypsin rendered the cells slightly more resistant to invasion. Pasvol *et al* (1982) also noted that once the merozoite had invaded these cells development of the parasite continued normally.

1.3.1.2.14 The Gerbich blood group antigens.

The Gerbich (Ge) blood group system is composed of a group of carbohydrate dependent antigens located on glycophorins C and D. They are defined by a series of antibodies that were originally described by Rosenfield *et al* (1960) in three patients from different parts of the world (New York of Italian extraction, Texas of Mexican origin and Århus Denmark respectively). In all

three examples the antibody was discovered when the babies of the patients concerned had positive direct coomb's tests on their cord blood samples and subsequent investigations demonstrated the antibodies in the mothers sera. In all cases the babies did not develop significant haemolytic disease of the newborn. Later more examples of the antibody were discovered and it became clear that at least 3 different antigens were involved and these subsequently became known as Ge:1, Ge:2 and Ge:3 respectively and since they all occur on almost 100% of all human red cells they are termed public antigens. The Ge:3 determinant is present on both GP C and GP D whereas GP:2 appears to be located on GP D only (Anstee 1990). The location of Ge:1 has not been determined since antisera to this is very rare. Both glycophorins appear to be the products of a single gene (2q14-21) (Tanner *et al* (1988). A mechanism by which the single gene codes for two proteins has been proposed by Tanner *et al* (1988). They suggest that scanning of the initiation sequence of mRNA (the first methionine codon (AUG) from the 5' end) by the 40S ribosome may not be absolutely efficient and initiation may only occur when the next AUG codon is encountered downstream. This is known as "leaky" initiation. The mRNA for GP C has an AUG sequence that would give rise to an amino acid at position 22. Should the first sequence initiator be missed the resultant protein arising from initiation at the AUG sequence at position 22 would be a truncated form of GP C. This is in keeping with the proposed sequence of GP D as suggested in a review by Blanchard (1990). It does not, however, account for the presence of the Ge:2 antigen at the N-terminal of GP D which is not expressed on copies of the full GP C. A possible explanation for this is that the sequence for Ge:2 is present

on GP C but is masked by the configuration of the terminal 22 amino acids which are not present on GP D.

The variant Gerbich types are determined in terms of their reactions with the 3 types of antibody and various patterns have been established.

	Antibody to			
	Ge:1	Ge:2	Ge:3	Genotype
Normal	+	+	+	Ge 1,2,3 (Ge2,3)
Gerbich (Ge) type	-	-	-	Ge-1,-2,-3 (Ge-2,-3)
Melanesian type	-	+	+	Ge-1,2,3 (Ge2,3)
Yus type	-	-	+	Ge-1,-2,3 (Ge-2,3)
Leach phenotype	-	-	-	Ge-1,-2,-3 (Ge-2,-3)

Table X Serology of the Gerbich phenotypes
(Since Anti Ge:1 is rare the phenotypes are often expressed only in terms of Ge:2 and Ge:3 and these are given in parenthesis) (Reid 1986)

The Ge and Leach phenotypes have the same serological reactions but are clearly distinguishable from each other on the grounds of red cell morphology since the erythrocytes of the Leach phenotype are elliptical. Red cells from all the other Gerbich phenotypes are normal morphologically although in some populations other independently heritable conditions which give rise to changes in cell shape may co-exist with Gerbich variants.

The defects which give rise to most of the various Gerbich negative states have been characterised (Chang *et al* 1991) the notable exception being the Ge-1,2,3 which is probably the commonest type in Melanesians. (Figure 7)

summarises the exon deletions on the GP C (ß) gene responsible for the various different phenotypes. The mechanism by which the exons are deleted in the Ge and Yus phenotypes as proposed by Chang *et al* (1991) involves a tandem duplicate sequence of 3.4kb each spanning exons 2 and 3. This may result in unequal crossover of homologous chromosomes with deletion of one exon. If the crossover is 5' to the misaligned exons then the Yus phenotype is produced with exon 2 being deleted (Figure 8). The Ge

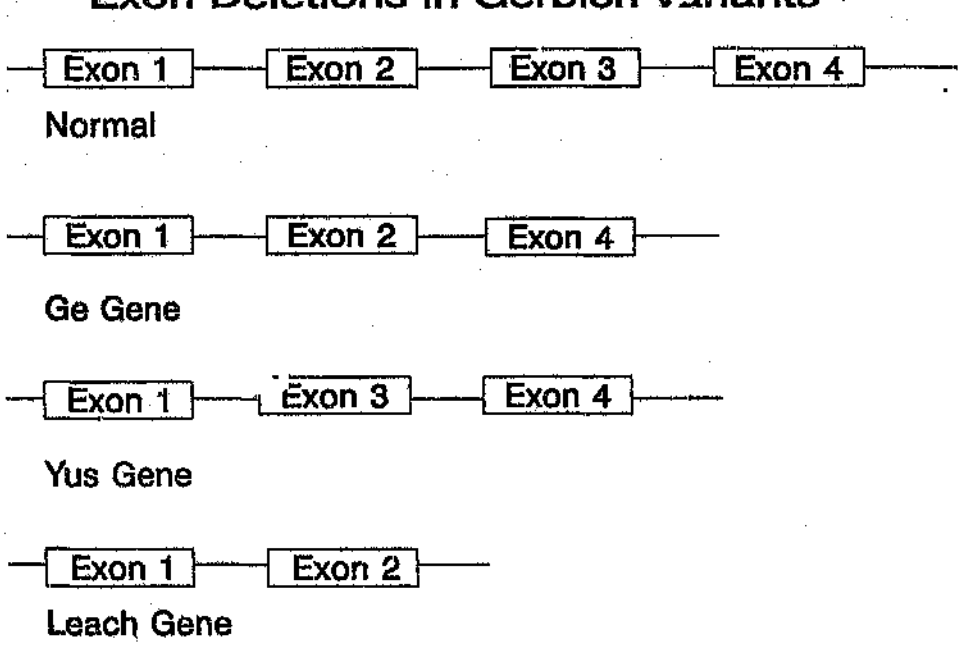


Figure 7 The gene deletions which constitute the various Gerbich genes.

phenotype is produced by a crossover 3' to the misalignment with deletion of exon 3 (Figure 9). The Leach phenotype represents a complete absence of both GP C and GP D although only exons 3 and 4 are deleted. The resultant protein lacks the normal intramembrane and cytoplasmic domains and therefore membrane insertion does not take place (Tanner *et al* 1988). Telen *et al* (1991) showed in one individual with this phenotype a single nucleotide

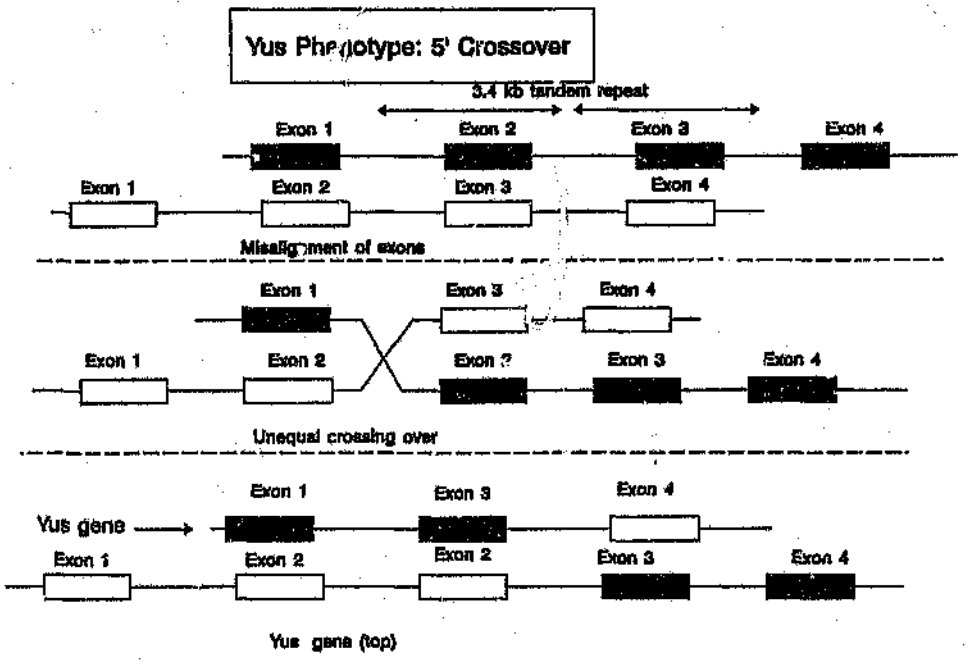


Figure 8 The molecular mechanisms of the exon deletions which result in the Yus gene.

deletion within exon 3 that produced a premature stop codon. This results in truncated GP C and GP D proteins which they presume are not inserted into the red cell membrane.

The Gerbich negative phenotypes are rare in most populations throughout the world with the exception of the Melanesians. In Papua-New Guinea certain ethnic groups have a high proportion of Gerbich negative individuals (Booth *et al* (1970) where gene frequencies for Gerbich negative (Ge-) of up to 0.80 have been observed. (Booth and McLoughlin 1972). Subtyping of the Melanesians has shown that most of the Ge- in this population are GE -1,2,3 which appears to be unique to this region. Some Melanesians are of the Ge phenotype (Ge-1,-2,-3) but no examples of the Yus or Leach type have been found.

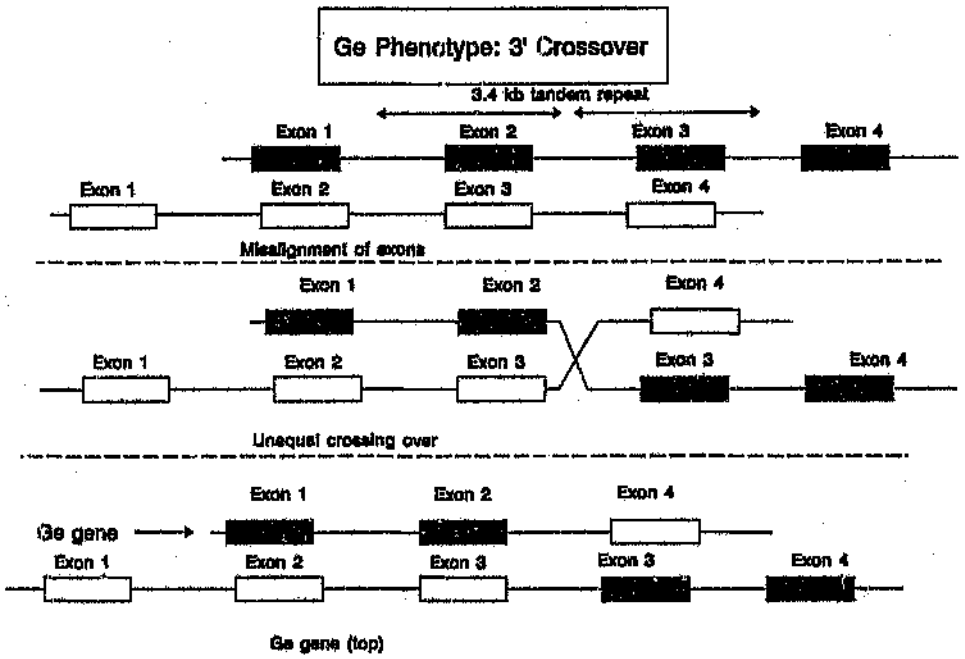


Figure 9 The molecular mechanisms of the exon deletions which result in the Ge genes.

Pasvol *et al* (1984) investigated the ability of *P.falciparum* merozoites to invade the elliptocytic red cells of a patient lacking GP C (presumably the Leach phenotype). This group found the parasite was able to invade the cells but with decreased efficiency (about 57% of normal). Serjeantson (1989) in a survey of the malaria endemic areas of the northern provinces of Papua New Guinea found a significantly smaller number of cases of infection with *P.falciparum* and *P.vivax* in Gerbich negative individuals than in Gerbich positive subjects. There was no difference between the two groups with regard to *P.malariae* infections. This worker suggests that the increased deformability of Gerbich negative red cells may be the mechanism of resistance and this may be a selective advantage in the malarious areas. Wesche *et al* (1990) (unpublished) showed that there was no significant

difference in the invasiveness of *P.falciparum* in an *in vitro* culture system. This is contrary to the epidemiological evidence of Serjeantson whose results are not entirely clear as she chooses to combine infections with 2 species rather than giving the individual data. It is also not clear as to the ovalocytic status of the patients used in the study. This is important since ovalocytosis which does confer innate resistance against *P.falciparum* (Kidson *et al* 1981) is also common in Melanesia and is due to a defect in band 3 (Liu *et al* 1990) and is independent from the Gerbich blood group system. This and other groups (Saul *et al* 1984, Mohandas *et al* 1984) have concluded that the increased rigidity of the membrane is the mechanism that blocks invasion by the malaria parasites which is in contrast to Serjeantson's view that increased deformability is a factor.

The Gerbich negative phenotype has been recorded in at least two South African families (Moores *et al* 1990). The first of these (Coet, a man of mixed ethnic origin) was phenotyped by Daniels (1982) as Ge:-2,-3 . The family described by Moores *et al* (1990), also of mixed ethnic origin, is most unusual in that in addition to being Gerbich negative (Ge:-2-3) the propositus also had Dantu positive erythrocytes.

1.4 Research objectives

This project had two main objectives. The first was to develop a reliable method of enumerating parasites in continuous culture. This is necessary in order to assess the rate of merozoite invasion in various cell types so that comparisons can be made. The flow cytometer appears to be the instrument

best suited to this task. Makler *et al* (1987) developed a method using thiazole orange (10^{-5}M) which is a fluorochrome excited at 488nm with an emission wavelength of 530nm. This is well suited to the instruments employing the argon ion laser. Makler's method was developed for the Becton Dickinson FACSCAN™ and needed to be adapted to the Coulter Profile II™ which is available locally.

The second aim of the project was to investigate the ability of *P.falciparum* merozoites to invade and develop in red cells with variant or polymorphic antigens present. The cells available for this included Henshaw, S-s-U- and the Dantu type hybrid. Unfortunately no antisera were available for typing cells for the Hunter antigen.

2 MATERIALS AND METHODS.

2.1 Blood Samples

Blood samples were drawn from random black blood donors who presented themselves for voluntary donation at bleeds organised by the South African Blood Transfusion Service (SABTS) or the Highveld Blood Transfusion Service (HBTS). Dantu cells were obtained by special arrangement from blood donors at the Natal Blood Transfusion Service, Durban and from the Provincial Tissue and Immunology Laboratory, Cape Town. A single sample of the S-s-U- phenotype was also supplied by the Natal Blood Transfusion Service, Durban. All samples were collected in Acid Citrate Dextrose (ACD) (Becton Dickinson Vacutainers™ 6ml).

2.2 Experiments

Several experiments were performed to assess the invasibility of *P.falciparum* merozoites into the red cells of the various polymorphic varieties that were available. In each case multiple cultures were done in 6 or 18 well micro culture plates as according to the Candle jar method of Trager and Jensen (1976) as described below. The inoculating cultures were all concentrated by the gelatine sedimentation method and the 20μl of the resultant concentrate of late trophozoites and schizonts were added to 10ml of a 1% suspension of the test cells and mixed well. This was then dispensed into the microculture plates in 1 ml aliquots (18 well plate) or 3ml aliquots (6 well plates). The initial dispensing of sub cultures into the microculture wells was done by a third party and the code only revealed at the conclusion of the experiment.

All the cultures and the associated techniques were carried out under a sterile laminar flow hood.

The cells were subjected to flow cytometry initially and thereafter every 24 hours for 72 hours. Cultures were not maintained beyond 72 hours since the limitation of nutrients and the metabolic products of the parasites produced erratic growth cycles which tended to become asynchronous. [H^3] hypoxanthine incorporation studies were also performed on parasites growing in Dantu and Henshaw positive cells and compared to normal controls run concurrently. All parasites were the FCR3 strain and were provided by the Department of Clinical and Experimental Pharmacology, University of the Witwatersrand.

2.3 Malaria Parasite Culture.

The method of Trager and Jensen (1976) as amended by Freese *et al* (1988) was used to culture the malaria parasites. The media and cells were prepared as follows:-

1. The "incomplete" medium was prepared by dissolving 10.4g of RPMI 1640 with L-Glutamine (Highveld Biochemicals), 5.94g *N*-2-Hydroxyethylpiperazine-*N*'-2-Ethanesulphonic Acid (HEPES), 4.0g Glucose and 44mg hypoxanthine were dissolved in 960 ml deionised autoclaved water and mixed with a sterile magnetic stirrer and then allowed to stand for an hour. Gentamycin Sulphate (50mg) was added and the mixture filtered through a Sterivex-GS® 0.22 μm filter

(Millipore®) in 90 ml aliquots into Schott bottles. This was stored frozen at -20°C.

2. The "complete" medium was made up by adding 10ml group AB pooled complement inactivated human plasma and 4.2 ml 5% Na_2HCO_3 to 90 ml of freshly thawed incomplete medium.
3. The red cells used to host the parasites were prepared from citrated whole blood by removing the plasma and buffy coat and washing twice in incomplete RPMI 1640 medium.

The parasites were maintained in continuous culture in 800ml flat bottomed (175 cm^2) culture flasks (Nunclon ® Δ) as a 1-5% red cell suspension in 25 ml complete RPMI 1640 medium in an atmosphere of 3% Oxygen, 4% Carbon Dioxide, 93% Nitrogen (Afrox). The medium was changed daily and red cells were added when needed to maintain a parasitaemia of about 5% and an haematocrit between 2 and 10%.

2.4 Micro Cultures

This method was used to do all the experimental work as it enables many cultures to be run in parallel. The total volume in each of the 18 wells was 1ml and parasites are best cultivated at an haematocrit of about 1%. The atmosphere of approximately 5% CO_2 was obtained by placing the plate in an unstoppered bell jar and burning 2 candles. Once a concentration of 5% CO_2 was reached the candles stopped burning and the jar was stoppered and placed in the incubator (37°C). The candle jar is illustrated in Plate 1.

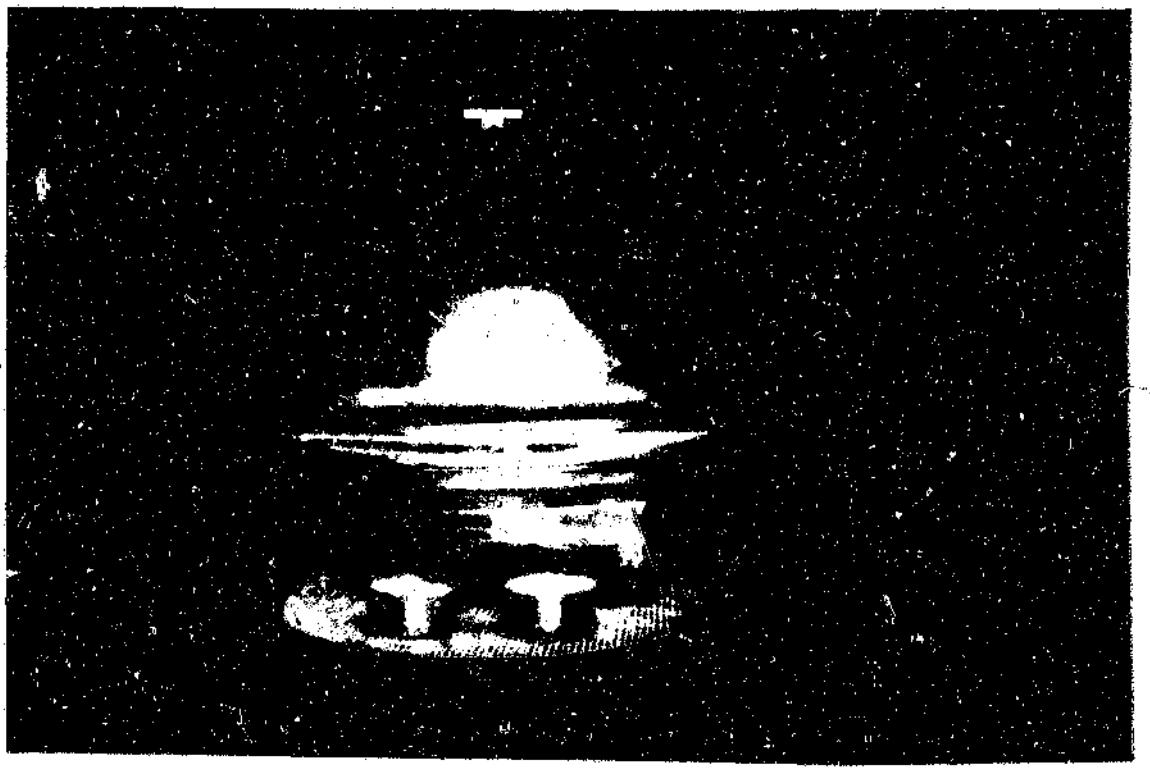


Plate 1 The candle jar used for the micro plate culture technique.

2.5 Gelatin concentration of parasites

The technique by Jensen (1978) was used to separate the mature trophozoites and schizonts from the earlier forms and the uninfected red blood cells. The concentrates of these late forms were used to prepare subcultures in all the experiments conducted. A micrograph of concentrated parasites is seen in Plate 2. 1g gelatin was dissolved in the 100 ml incomplete RPMI 1640 by heating to 56°C and was sterilised by filtering through a Sterivex-GS® 0.22 μm filter (Millipore®). This was stored as a gel at 4°C and warmed to 37°C to liquify for use.

The parasites were concentrated as follows

1. 4 ml of packed red cells were added to 6ml of the warmed 1% gelatin solution and 24 ml incomplete RPMI 1640.
2. This was mixed well and centrifuged for 5 minutes at 400g and the supernatant discarded.
3. 10 ml warmed gelatin solution and 10ml incomplete RPMI 1640 was added to the pellet of cells and mixed well and equal volumes were dispensed into 2 x 15 ml plastic centrifuge tubes and placed upright immersed in water at 37°C.
4. The cells were allowed to sediment for approximately 30 minutes.
5. Sedimentation was deemed to have occurred when two distinct phases became visible. The schizont and late trophozoite infected cells in the upper phase were collected into a separate centrifuge tube and centrifuged at 400g and the supernatant discarded. The pellet, rich in schizonts and late trophozoites, was used to inoculate subcultures for the experimental work.

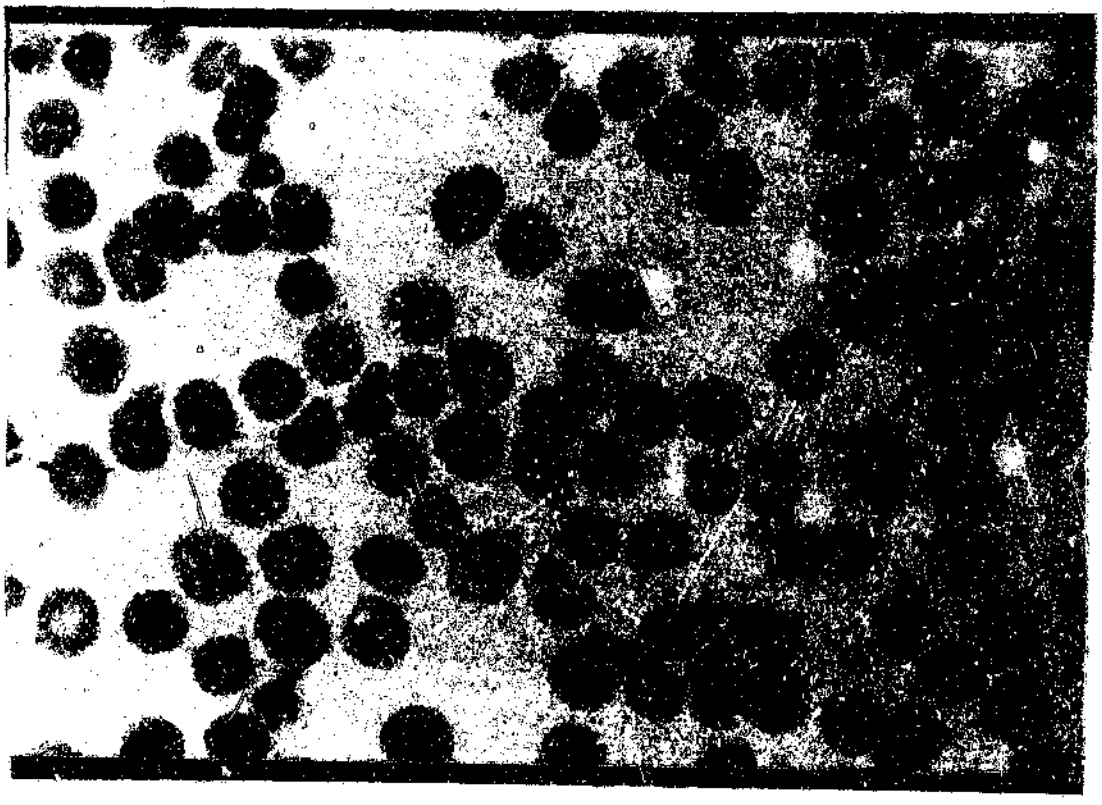


Plate 2 Parasites after concentration by the gelatin sedimentation method.

the experimental work.

2.6 Sorbitol synchronization of parasites in culture.

P.falciparum parasites in *in vitro* continuous culture loose the normal synchronicity of their cycles that is shown *in vivo*. Sorbitol selectively lyses red cells parasitised with the mature forms. The non parasitised and ring form parasitised erythrocytes are left intact. The method of Lambros and Vanderberg (1979) was used to synchronise the parasites in this project.

1. The contents of a culture flask (approximately 28 ml) was centrifuged at 400g for 5min and the supernatant discarded.
2. The pellet was resuspended in 5 times its volume of 5% D-sorbitol.
3. After 5 minutes the suspension was centrifuged at 400g and the supernatant discarded and replaced with complete RPMI 1640 medium. The desired haematocrit of between 1 and 5% was obtained by the addition of fresh red blood cells.

2.7 Giemsa staining of blood films

Working Giemsa stain was prepared immediately prior to use by diluting 1ml stock solution to 10ml with Phosphate buffer pH 7.2.

The thin blood films were air dried and fixed with methanol for 20 seconds and allowed to dry. They were then placed on a staining rack and flooded with working giemsa stain for 15 minutes. They were washed in running water blotted dry and examined under a 100X oil immersion lens on the microscope (1000x magnification). The parasitaemia was estimated by counting 1000

cells in the well spread areas of the slide. The morphology of the parasites was ideally studied by this method. The nucleus of the parasite stained red and the cytoplasm blue. Malaria pigment stained poorly and remained its native brown colour.

2.8 Flow cytometry

Flow cytometry, using thiazole orange to stain the parasites, was performed on the Epics profile II flow cytometer.

1. Stock thiazole orange (2.1mM) was prepared by dissolving 1mg Thiazole orange (MW 476) in 1ml of analytical grade Methanol. This was stored in the dark at -20°C.
2. Working thiazole orange was made up by adding 0.5µl stock thiazole to 1ml Phosphate Buffered Saline for every test to be performed just prior to use.
3. For each sample 1ml working thiazole orange was dispensed into 75 X 12mm glass test tubes and 2µl of parasitised red cells were added to this solution. A negative control was prepared by the identical technique but 2µl non parasitised cells were used.
4. The tubes were incubated in the dark for 1 hour at room temperature and flow cytometry performed.

The Epics® Profile II flow cytometer (Coulter Corporation) was set up as in Table XI and the histograms as according to Table XII. The target red cell population was bitmapped as seen in Figure 10 to exclude any leucocytes

which may interfere with the analysis. Figure 10 also shows examples of the other histograms and with the data used to generate histograms 2 and 3 being derived from the bitmapped area in histogram 1.

Parameter	Setting	
Sample Volume	200 µl	
Sample Flow Rate	10 µl/min	
Sheath Pressure	13.20 psi	
Laser Power	15mW	
PMT Voltages		
Side Scatter	300v	
Fluorescent 1	1000v	
Fluorescent 2	0v	
	Gains	Discriminators
Forward Scatter	50	100
Log Side Scat	1	1023
Log Fluorescent 1	1	1023

Table XI The set-up parameters for the Epics™ Profile 2 flow cytometer for the estimation of malaria parasites

HISTOGRAM NUMBER	Y AXIS	X AXIS	Gating	Scaling
1	Forward Scatter	Log Side Scatter	Area Bitmapped [1]	100
2	Forward Scatter	Log Side Scatter	In Gating Bitmap 1	40
3	Count	Log Fluorescent 1	In Gating Bitmap 1	20-50
4	Count	Forward Scatter	No Gating	Auto Scale

Table XII The histograms used in the flow cytometric estimation of Malaria parasites *in vitro* culture.

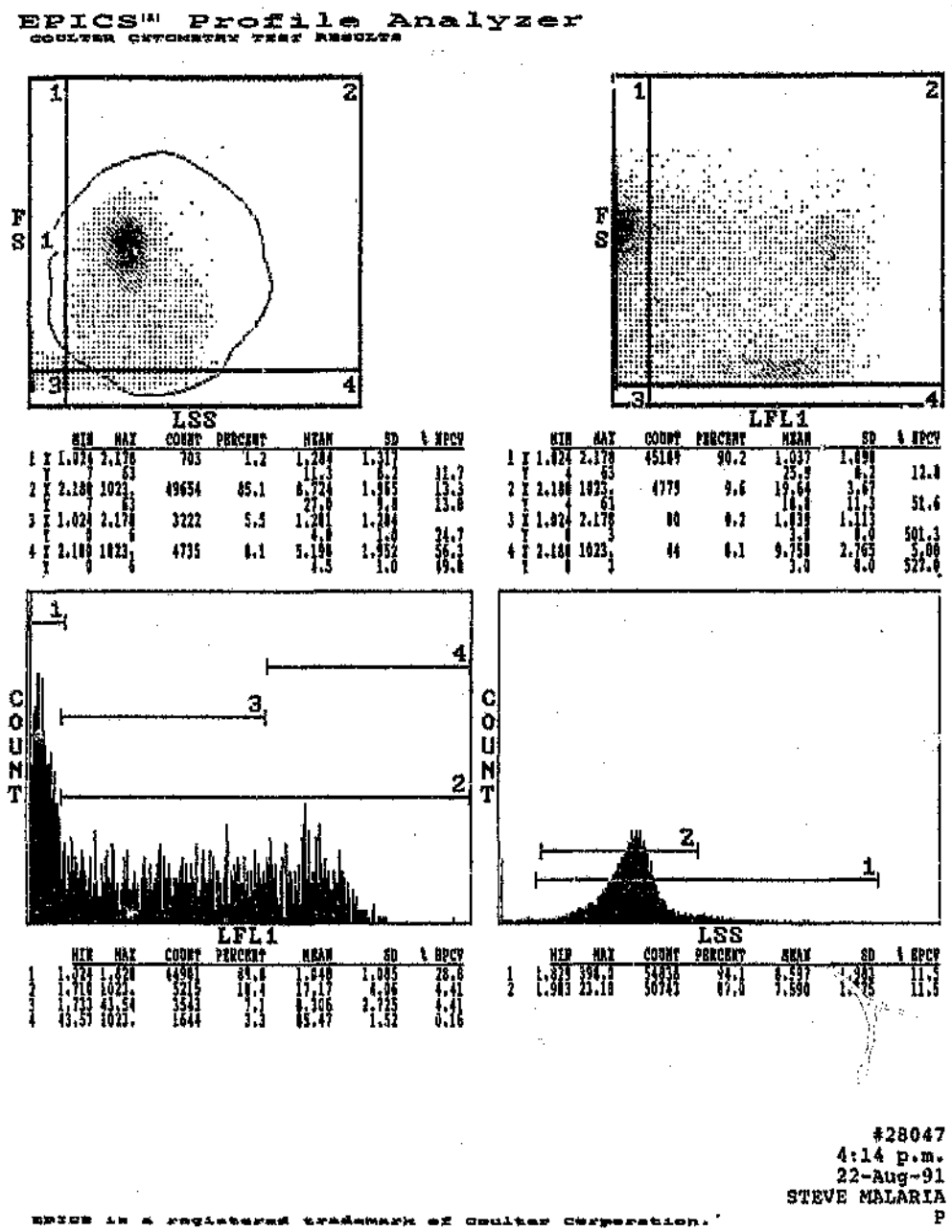


Figure 10 The histograms used for the enumeration of malaria parasites on the Epics® Profile flow cytometer

2.9 [H^3] hypoxanthine incorporation

The measurement of [H^3] hypoxanthine incorporation was performed to assay the invasability of merozoites in a single parasite cycle. The technique used was that described by Chulay *et al* 1983). The incorporation of [H^3] hypoxanthine as expressed in counts per minute (cpm) is directly proportional to the number of merozoites successfully invading the red cells under test. Providing the starting parasitaemias are approximately equal this technique can be used to compare the invasion rates of parasitaemia into different cell types.

1. Stock G-[H^3] hypoxanthine (approximately 1.0 mCi.) (Amersham) was made up by reconstituting the lyophylate with 2ml 50% ethanol. This was stored at -20°C.
2. Working G-[H^3] hypoxanthine was made up just prior to use by pipetting 200μl of the stock solution into a sterile tube. The ethanol was evaporated off under a stream of Nitrogen and the residue was dissolved in 2.5ml hypoxanthine free complete RPMI 1640 medium. (this gave a final activity of approximately 40μCi/ml RPMI 1640).

Cultures were prepared for the assay by concentrating parasites from the stock culture by the gelatin flotation method. A giemsa stained blood film was examined to ensure that an adequate yield of late trophozoites and schizonts had been obtained and the parasitaemia estimated. A calculated volume of the concentrate was added to a 1% suspension,in hypoxanthine free complete RPMI 1640, of the red cells under investigation to give a parasitaemia of approximately 1%. A control red cell suspension was prepared using the

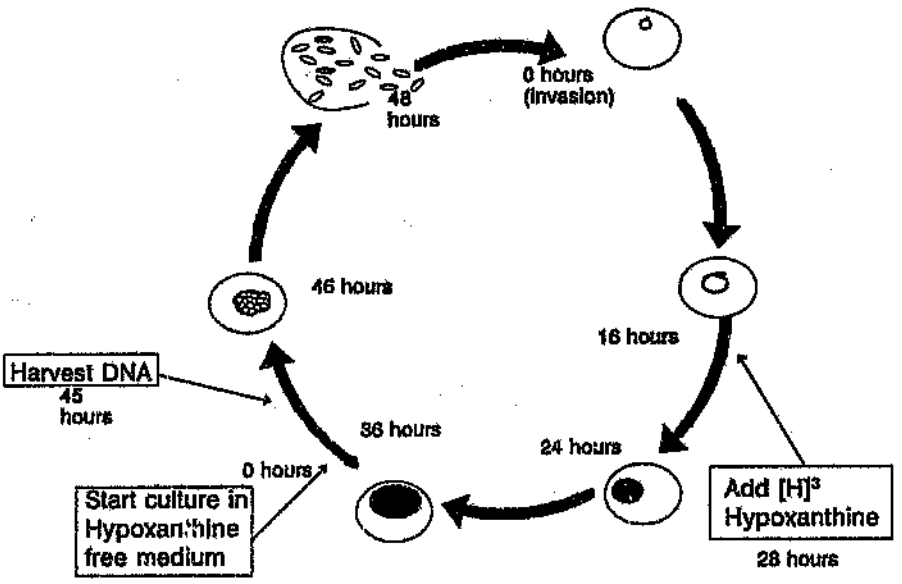


Figure 11 The erythrocytic life cycle of *P.falciparum* showing the stages involved in the [H^3] hypoxanthine incorporation test.

same cells but without parasites. 175μl is of the respective cell suspensions were dispensed into a flat bottomed microtitre culture plate with each cell type and the control being assayed in multiples of 12 (ie 1 row). The plate was incubated at 37°C for 24 hours in a candle jar. After 24 hours the hypoxanthine free complete RPMI 1640 is removed by suction and replaced. A slide is prepared from one of the wells and the stage and number of the parasites noted. 25μL Working G-[H^3] hypoxanthine is added to each well when it is estimated that the parasites are at the "16-18 hour" stage (young ring forms are at the "0 hour" stage - see Figure 11). The culture was returned to the candle jar and reincubated for 26 hours. The parasites were harvested on a Titertek ™ Cell Harvester (Flow Laboratories) by washing the cells in deionised water (which lysed the cells) and passing the washings through Whatman ™ glass microfibre filter under gentle negative pressure. The parasite DNA adhered to the glass filter whereas the free hypoxanthine was washed away. Pressed filter discs (each corresponding to a culture well)

were removed from the harvester and dried at 60°C for 20 minutes. The disks were then placed in scintillation counter bottles and 5ml of Aquagel 1™ added to each bottle which were then capped and counted on a ß Scintillation counter for 1 minute. The counts, expressed as counts per minute, were then corrected for background by subtracting the mean of the blank from each of the test assays. The mean of the corrected counts for each red cell type under test was calculated and a statistical evaluation done to detect significant differences between the groups.

2.10 Blood grouping

The cells used in this study were typed for the M,N,S,s and Henshaw blood groups. The U group was done on any cells that typed negative for S and s.

Cells were prepared for testing by adding 2 drops of red cells to a 75 x 12mm test tube and topping up with 0.9% saline. The cells were washed once by centrifuging at 400g for 1 minute. The saline was decanted rapidly and the cells resuspended. A volume of saline was added so as to make a final suspension of 3-4%.

2.10.1 M and N groups

The reagents used to type blood cells for the M and N antigens were manufactured by Gamma Biologicals Inc. Houston Texas (Gamma-Clone® murine monoclonal anti M and anti N). The method of testing as recommended by the manufacturer in their package insert was followed.

One drop of an approximate 3-4% suspension of red cells was added to one drop of Gamma-clone anti-M or anti-N in a labelled test tube (75 x 12mm) and mixed thoroughly. This was incubated for 15 minutes at room temperature (23°C ± 3°C). This was centrifuged for 15-30 seconds at 700g in a Dade Immunofuge®. The cells were resuspended and the tubes were read macroscopically for agglutination and if positive graded according to the nomenclature shown in Table XIII .

2.10.2 S,s and U groups

The reagents used to type blood cells for the S,s and U antigens were commercially manufactured by Gamma Biologicals Inc. Houston Texas (Gamma blood grouping reagent anti-S, anti s and anti U). The method of testing as recommended by the manufacturer in their package insert was followed.

One drop of an approximate 3-4% suspension of red cells was added to one drop of the appropriate Gamma-clone anti sera in a labelled test tube (75 x 12mm) and mixed thoroughly. The tubes were then incubated for 15-30 minutes at 37°C. The cells were then washed 4 times with saline. Two drops of polyclonal anti Human Globulin was added to the washed button of cells, mixed and centrifuged 1 minute at 100g. The cells were resuspended and read macroscopically for agglutination and the reaction graded.

2.10.3 Henshaw

The reagent used for Henshaw typing was supplied by the Natal Blood Transfusion Service. The method of testing was as for anti-M and anti-N as

described above but the incubation period was 30 minutes at room temperature.

2.10.4 Controls

All the above described procedures were controlled as follows:

The antibody identification panel A as supplied by the Natal Blood Transfusion Service was used a source of positive and negative controls for the anti-M,anti-N,anti-S,anti-s and anti-U reagents. The Henshaw reagent was controlled with strongly positive and weakly positive Henshaw cells supplied by the Natal Blood Transfusion service. The antiglobulin test (Coomb's test) was controlled with group O positive cells sensitized with anti-D

GRADE	DEFINITION
4	Complete Agglutination
3	Large separate masses of agglutinates
2	Smaller agglutinates easily visible
1	Very small clumps which give a grainy appearance macroscopically.
-	Negative.

Table XIII Grading nomenclature used for blood grouping reactions.

3 RESULTS

3.1 Evaluation of the Flow cytometer and Culture systems.

3.1.1 Precision of flow cytometry and manual estimates of parasitaemia.

The precision of the flow cytometric enumeration of malaria parasites was found to be superior to the microscopic method. The coefficients of variation in specimens reassayed 10 times on the flow cytometer had co-efficients of variation ranging from 6.52% at low parasitaemia down to 2.28% at a high parasitaemia. A single sample diluted 10 times has a co-efficient of variation of 6.74%. Microscopic counts yielded co-efficients of variation some 8 to 10 fold higher at a parasitaemia of approximately 2%. This data is summarised in Table XIV and Table XV.

	1 sample Diluted 10 times	1 Dilution assayed 10 times			
		LOW RANGE	MID RANGE	HIGH RANGE	UPPER RANGE
Mean	7.33	0.92	4.52	9.52	11.92
CV	6.74	6.52	5.75	2.77	2.28

Table XIV Precision of flow cytometry estimation of total parasitaemia based on counts of 10 000 cells.

Specimen number	Manual counts (5 technologists)					Statistics manual counts	
	1	2	3	4	5	Mean	CV (%)
1	1.8	6.0	4.6	5.0	11.2	5.72	53.74
2	1.8	3.8	2.6	6.0	9.0	4.64	56.03
3	1.4	2.6	2.8	3.8	12.0	4.52	84.45
4	2.4	4.4	5.0	4.4	17.8	6.80	81.21
5	1.6	2.4	2.8	6.0	10.4	4.64	69.95
6	2.8	2.8	2.6	3.4	8.4	4.00	55.41

Table XV Precision of manual estimation of parasitaemia based on counts of 500 cells.

3.1.2 Correlation of manual and flow cytometry estimations of parasitaemia.

The regression plot of manual counts (mean of 5 technologist's counts) versus flow cytometry is shown in Figure 12 below. The correlation between the 2 methods was 0.322 (Pearson's r) with the regression equation being:-

$Y = 0.291x + 1.727$ where Y is the flow cytometry value and x is the manual method value. Statistical analysis of the data showed there was no significant difference between the 2 data sets ($t=0.68$ $p= 0.533$).

A second assessment was made whereby an experienced malariologist assessed the parasitaemia of 12 slides and this was compared to flow cytometric analysis. The regression plot is seen in Figure 13. The correlation coefficient (r) is 0.311 with the regression equation being:-

$Y = 2.26X + 0.230$ where Y is the flow cytometry value and X is the manual method value. There was no significant difference between the 2 sets of data ($t= 1.035$ $p= 0.325$)

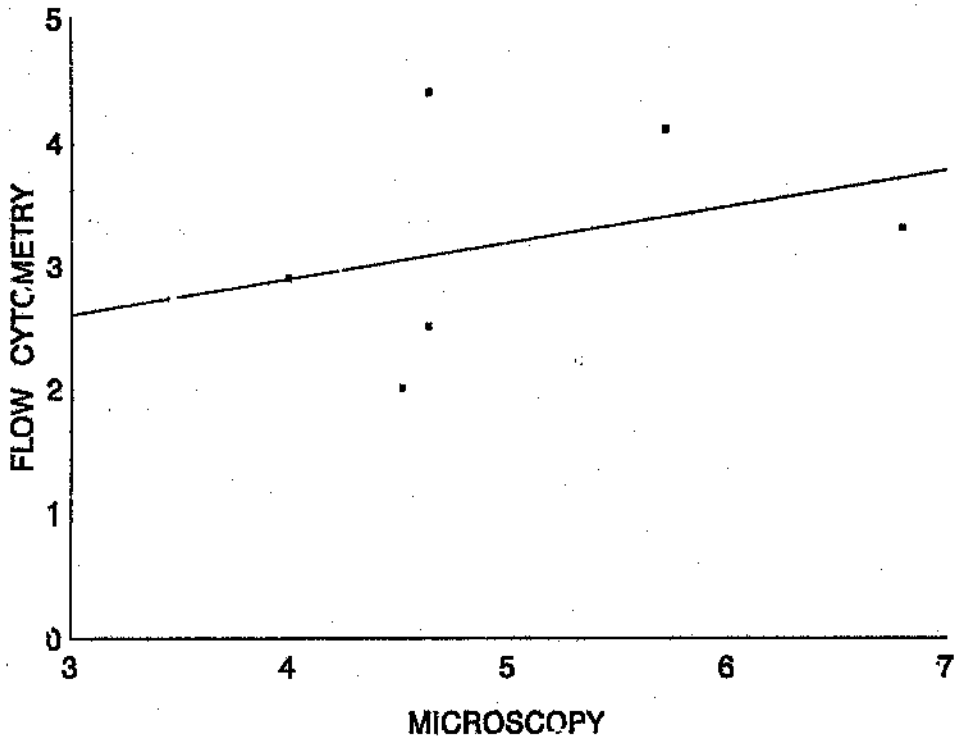


Figure 12 Correlation between microscopy and flow cytometry (see text)

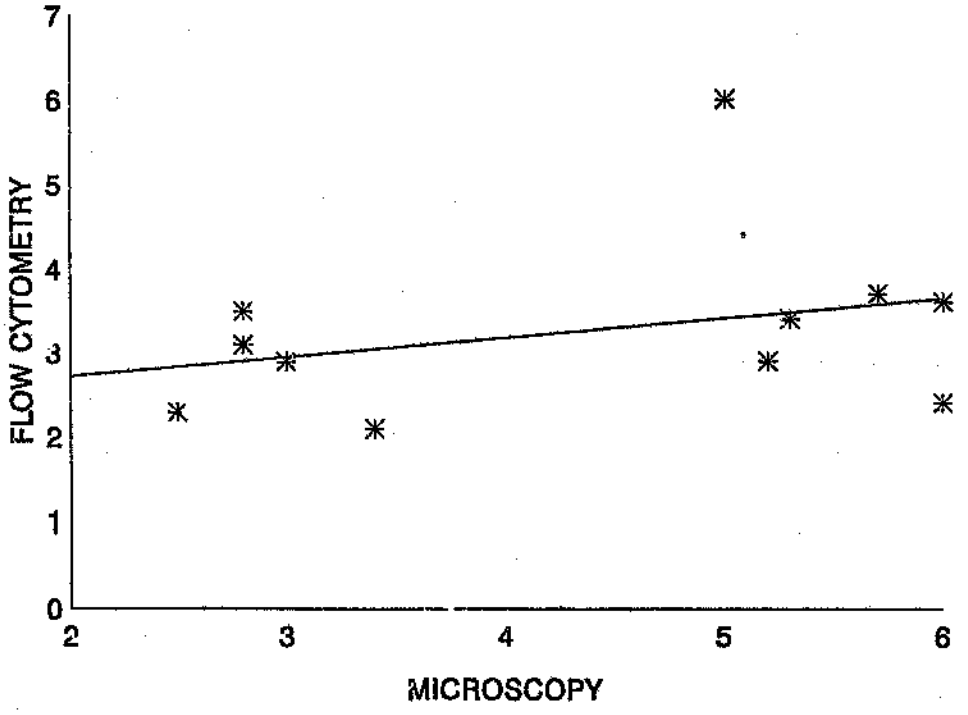


Figure 13 Correlation between microscopy and flow cytometry (see text)

Manual counts are done at the rate of 6 samples per hour whereas flow cytometry can be performed at 30 analyses per hour.

Serial cultures of the parasites in normal cells have a characteristic pattern of parasite incremental increases per 24 hours. Figure 14 demonstrates the erratic growth patterns beyond 72 hours which occur as a result of increasing amounts of metabolic products and substrate limitation as the parasitaemia increases. It is for this reason that the cultures used to evaluate the growth of parasites in the various cell types were terminated at 72 hours.

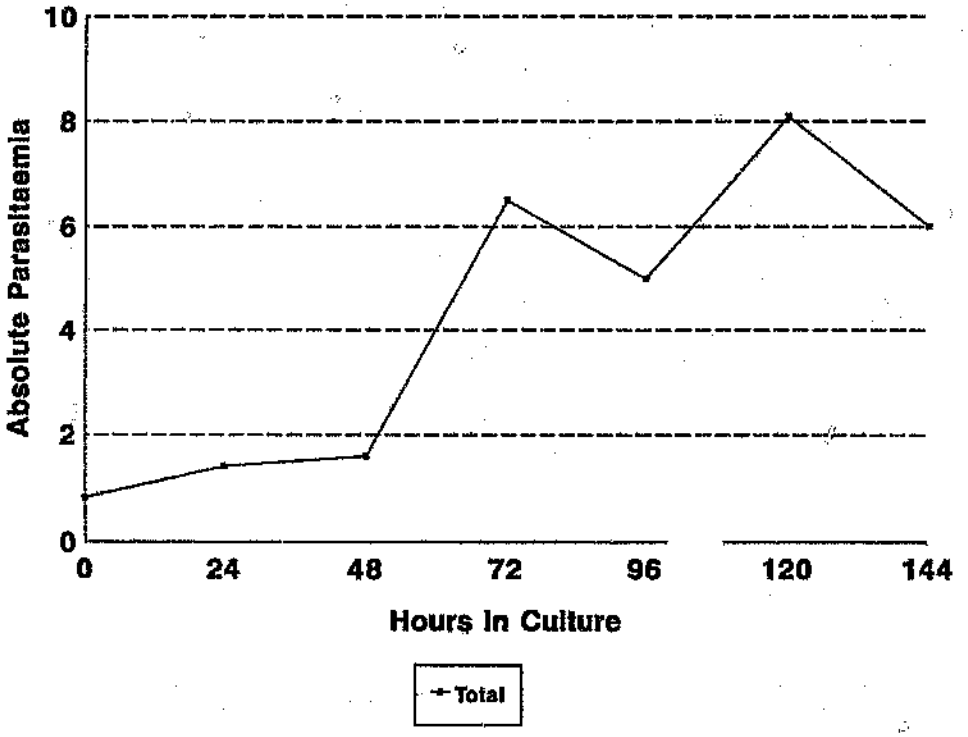


Figure 14 Serial culture of *P.falciparum* in culture using the flow cytometer to estimate parasitaemia.

3.2 Henshaw vs control

A total of 399 unonymous black blood donors in the Witwatersrand area were tested for the Henshaw antigen and of these 37 were found to be positive (9.27%). This gives a gene frequency of 0.0475. The cells selected for the malaria studies were also found to carry the U antigen and either or both of the S and s antigens. Only strongly reacting (grade 4) Henshaw positive red cells were used in this study.

The serial cultures of the Henshaw cells as compared to control are shown in Figure 15 and the [H^3] hypoxanthine incorporation in Figure 16. No statistically significant differences between growth of parasites in control or Henshaw cells

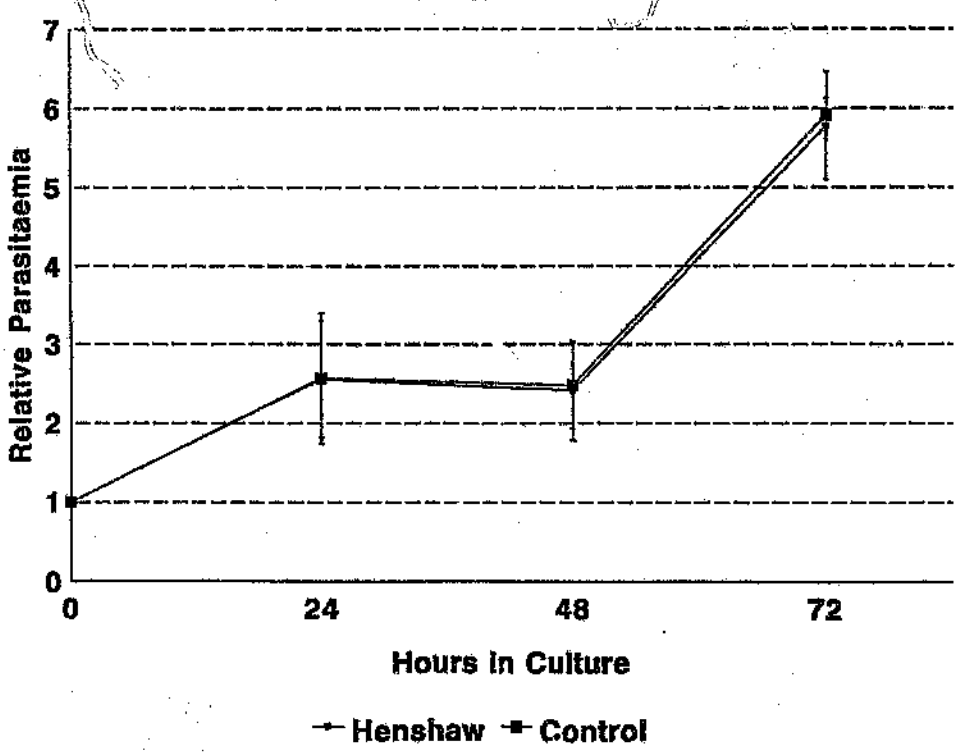


Figure 15 Henshaw vs control

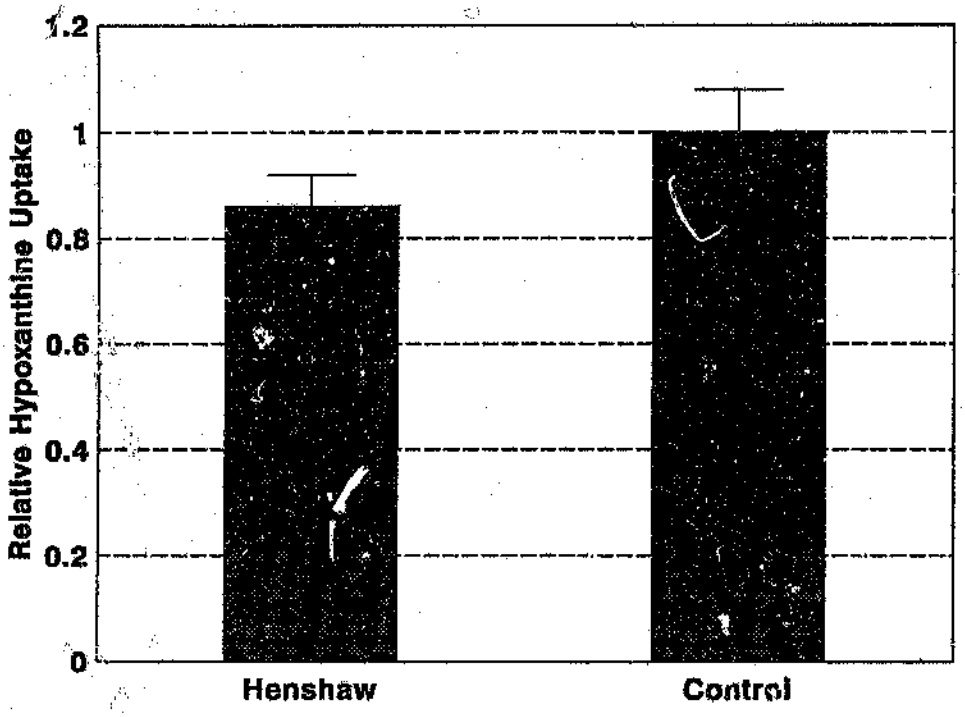


Figure 16 [H^3] hypoxanthine incorporation Henshaw vs control

could be detected at any of the time points. Statistical analysis was done by the Mann-Whitney test and expressed as the probability at time x (p_x)

In the case of the comparison of Henshaw and controls the probabilities were calculated to be

$$p_{24} = 1.00,\ p_{48} = 0.81,\ p_{72} = 0.14$$

Similarly there was no significant difference in the uptake and incorporation of [H^3] hypoxanthine between parasites cultured in Henshaw positive and Henshaw negative cells ($p=0.16$). In this instance Student's t test was used to compare the data.

3.3 S-s-U- vs control

The red cells used in this experiment were provided by the Natal Blood Transfusion Service and typed as:-

M-,N+,S-,s-,U-.

The serial cultures using these cells (Figure 17) showed statistical significant differences from controls.

p_{24} =2.17 x 10^{-4}, p_{48}= 6.15 x 10-4, p_{72} = 3.5542 x 10^{-5}

Since these cells were in short supply [H^3] hypoxanthine incorporation was not done.

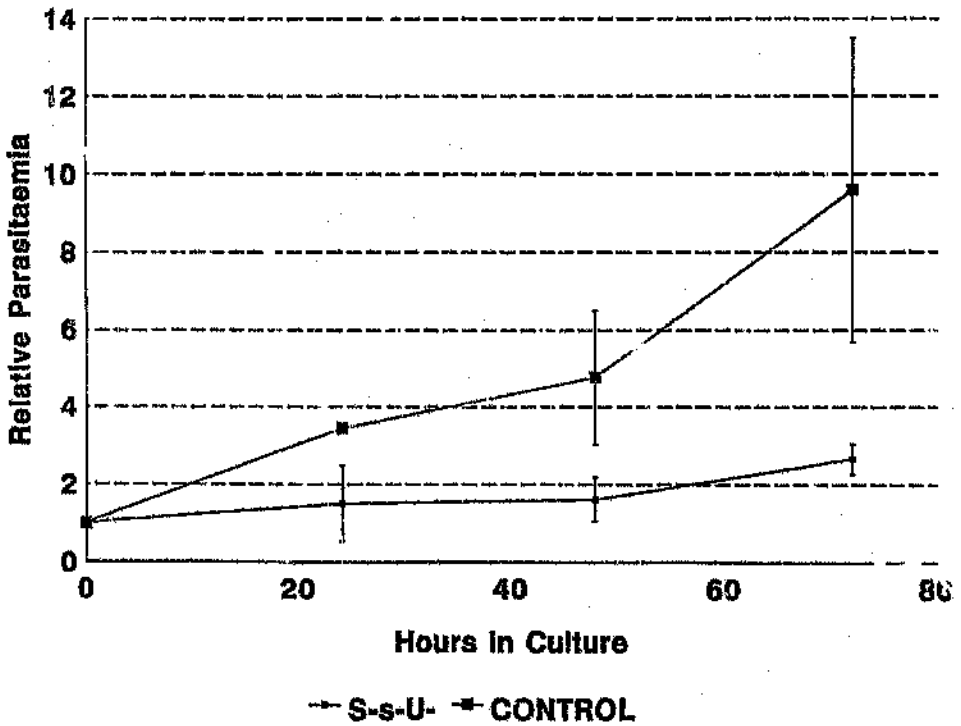


Figure 17 S-s-U- vs control

3.4 Dantu vs control

Dantu cells were provided by the Natal Blood Transfusion, Durban and the Provincial Laboratory for Tissue Immunology, Cape Town. The Dantu positive cells used in the various experiments typed as follows.

	Experiments performed	M	N	S	s	U	He	Dantu
1	Serial culture	+	±	-	+	+	-	+
2	Serial Culture	+	+	-	+	+	-	+
3	Serial Culture [H^3] hypoxanthine incorporation	+	+	+	±	+	-	+
4	[H^3] hypoxanthine incorporation	+	+	-	±	-	-	+

± = variable according to reagent

Table XVI The MNS blood groups of the Dantu cells used in this report. (Moores 1992, du Toit 1992).

Studies using Dantu cells in serial culture (Figure 18) have shown that the parasitaemia was significantly lower than in normal controls.

$p_{24} = 3.68 \times 10^{-3}$, $p_{48} = 0.014$, $p_{72} = 1.10 \times 10\text{-}5$.

[H^3] hypoxanthine incorporation (Figure 19) is 42% of that of normal controls after 1 cycle. The difference was found to be statistically significant on both occasions that it was estimated ($p_1 = 4.4 \times 10^{-6}$, $p_2 = 3.66 \times 10^{-7}$).

A most unusual feature was demonstrated on the stained films of parasites within the Dantu cells. The schizonts produced in Dantu cells appear to only have 10 -12 merozoites as compared to the normal 16 as seen in the control cells. The number of merozoites in a total of 30 schizonts in Dantu and control cells respectively were counted.

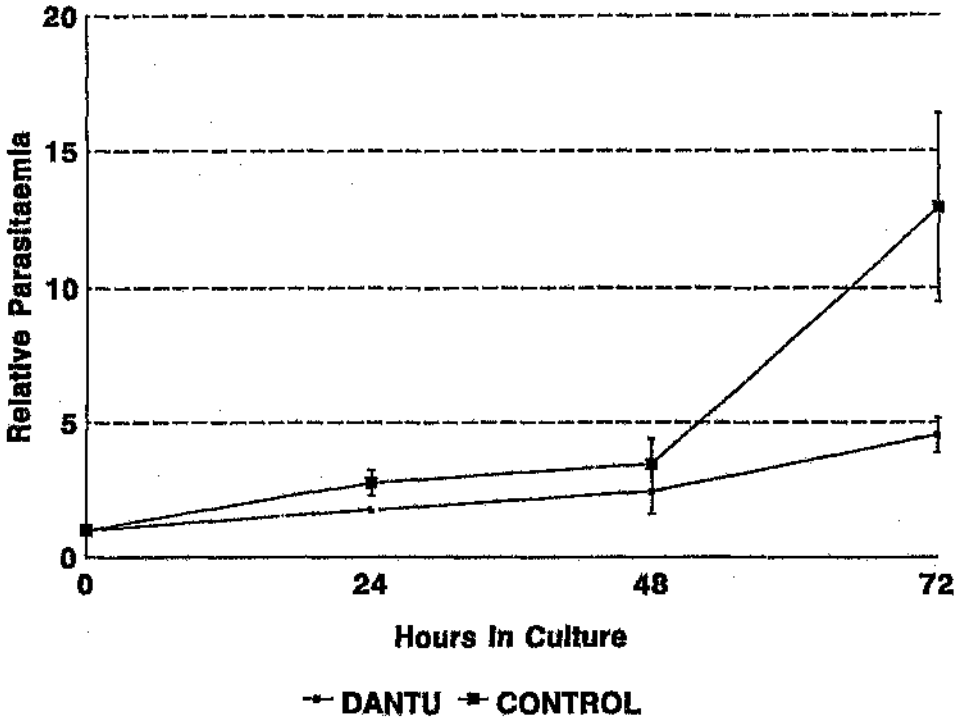


Figure 18 Dantu vs control

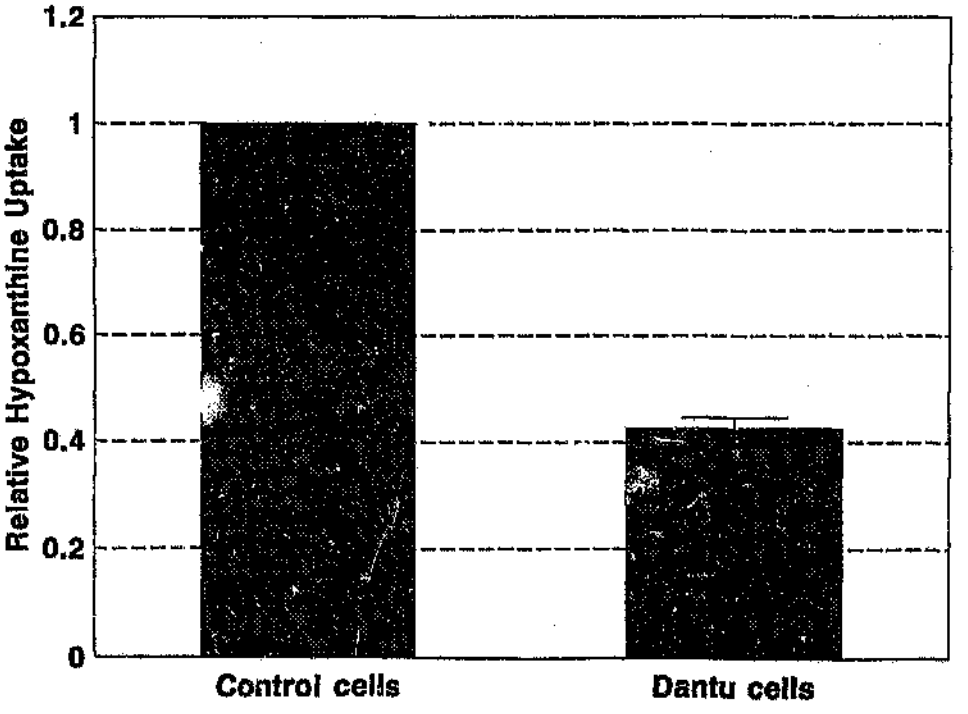


Figure 19 [H^3] hypoxanthine incorporation Dantu vs control

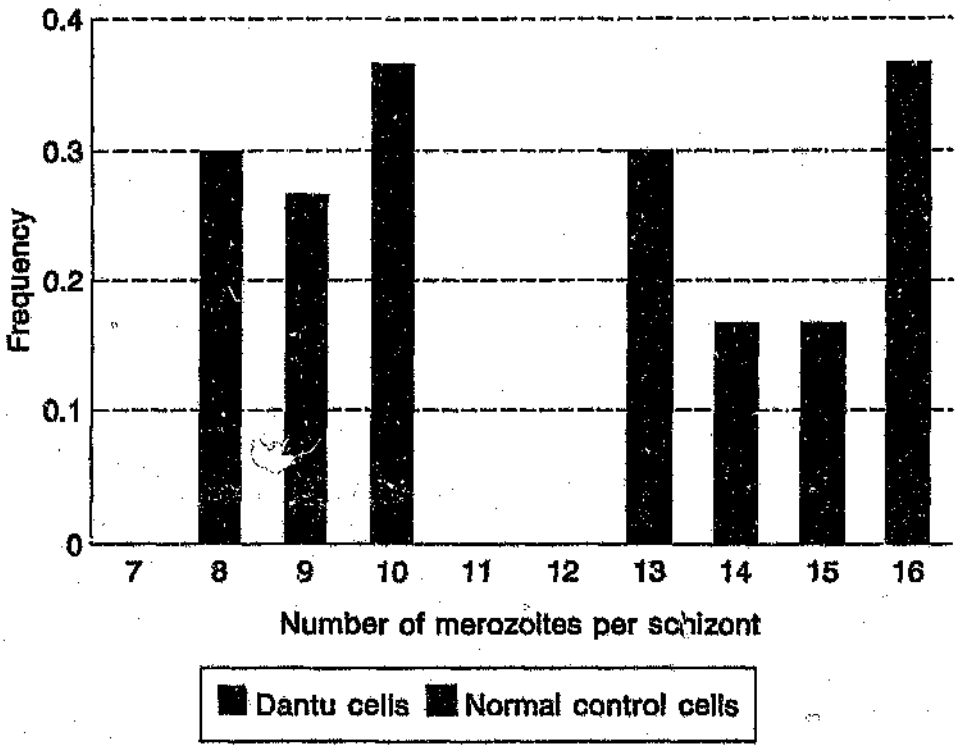


Figure 20 Frequency histogram of merozoite numbers in Dantu and control cells respectively.

The number of merozoites per schizont in the Dantu red cells ranged from 8 to 11 whereas in the control cells the range was from 13 to 16. The difference between the means of the two sets of data was statistically significant ($t = -18.5, p = 5.06 \times 10^{-26}$) Examples of these schizonts can be seen in Plates 3 and 4.



Plate 3 Schizont in Dantu Cell



Plate 4 Schizont in Control Erythrocyte.

4 CONCLUSIONS & DISCUSSION

4.1 Evaluation of flow cytometric analysis of malaria parasites.

The use of flow cytometry to count parasites grown in an *in vitro* culture system has added a degree of precision evaluation of cultures which cannot be achieved by microscopy. The microscopic examination of slides is of great value, and indeed cannot be replaced, in studying the morphological characteristics of the parasites in their various stages. There are, however, limitations to the ability of a microscopist to produce an accurate count as a result of intrinsic shortcomings in attaining a uniform distribution of parasites on the blood smear. The other major advantage of the flow cytometer is that the precision is greatly improved by the large number of cells it can examine over a short period whereas the microscopist is restricted to much smaller numbers and in large series the observer is subject to fatigue. The partitioning of the flow cytometry histograms into low fluorescence (representing the early stages) and high fluorescence (late trophozoites and schizonts) is useful but the figures cannot be regarded as absolute since the allocation of the channels is arbitrary. Further work on a sorting flow cytometer is required to establish the true partition parameters.

The flow cytometry technique also has the potential to be used in two colour studies whereby the parasite is stained with one dye (thiazole orange) and a membrane antigen is tagged with another (for example rhodamine) which emits light at a different wavelength to the first. This would enable direct comparison of a mixture of cells with and without the antigen under study. This was not done in this study because of the unavailability of anti Dantu and

a suitable fluorescent labelled anti rabbit IgM required to label the Henshaw cells.

4.2 Southern African blood groups and Malaria.

The presence of the Henshaw antigen on red cells does not appear to have been selected as a result of resistance to malaria. Although the Henshaw antigen is located on GP B its glycosylation is identical to that of He negative subjects. Thus the finding that the parasite invades He cells no differently from controls is consistent with the findings of other workers (Cartron *et al* 1983) and therefore supporting the contention that the sugar side chains are the important components on the glycophorin molecule for successful merozoite invasion. It remains an enigma as to how this antigen arose and which environmental factors (if any) which maintain the gene in polymorphic frequencies.

The findings with respect to the S-s-U- phenotype confirm the findings of Pasvol (1982) and Facer (1983). These results, therefore, were a good control in this study of a cell type in which invasion is impeded. This phenotype is also of interest as the gene deletion may also be found in association with some of the Dantu variants.

The Dantu phenotypes have not reached, and indeed are far removed from, polymorphic frequencies in Southern Africa. However the observation that *P.falciparum* invades these cells with difficulty would suggest that the

frequencies may be higher in the endemic areas of Africa to the North of the region studied in this report. Since the Dantu^{Ph} variant has been associated with a deletion of the S^U gene as well as the anti Lepore type formation of the δ-α hybrid (Huang *et al* 1988) it may be argued that frequencies of Dantu may reach polymorphic frequencies in similar regions to high incidences of the S^u gene. There are two possible reasons for this, the first being the powerful selective force of malaria operating in the region which would independently select both phenotypes. The other reason for the coexistence of both genes is that malalignment of the glycophorin genes in the formation of the hybrid may be facilitated by the deletion of all or part of the GP B gene on one of the chromosomes involved.

The mechanism of resistance of the Dantu cells to *P.falciparum* merozoites is most likely due to inability of the parasite to utilize the abnormal glycophorin as a receptor. As the cells do contain normal copies of GP A albeit in reduced amounts invasion is not completely blocked. The production of schizonts containing less than the normal 16 merozoites is an interesting observation and cannot be explained simply in terms of an abnormal membrane receptor inhibiting invasion of the parasite.

Growth of *P.falciparum* in red cells containing abnormal membrane proteins was studied by Schulman *et al* (1990). This group showed normal invasion of *P.falciparum* by [H^3] hypoxanthine incorporation and a normal pattern of growth over the initial 24-48 hours in cells from seven subjects with hereditary spherocytosis (HS). After 48 hours the growth in six of the HS subjects

showed a decline in the parasitaemia whereas the parasitaemia in controls continued to increase up to the conclusion of the experiment (144 hours). All the cells producing the abnormal growth pattern were spectrin deficient. The graphical representation of the data of Schulman *et al* (1990) bears a striking resemblance to that presemed in this report with reference to Dantu cells. However unlike spectrin deficient cells undergoing invasion by *P.falciparum*, the parasite in the infected Dantu cells incorporates less [H^3] hypoxanthine implying that invasion is less efficient in the latter cell type. The growth pattern in spectrin deficient cells is interpreted by Schulman's group as abnormal development of the parasite in its intracellular environment but they do not provide any morphological evidence to support this. They speculate that the parasite requires a normal host cell cytoskeletal proteins for normal development in the erythrocytic stage. A suggested mechanism is that the parasite utilises the host membrane proteins in the production of its own membrane. An alternative suggestion provided by these authors is that an interaction may occur between the host and parasite during intraerythrocytic growth.

It is not obvious how either of these models can be applied to the impairment of growth in Dantu cells since the division of the trophozoite in the formation of the schizont appears to be defective. It may be that in the case of spectrin deficient cells a similar phenomenon occurs which would account for the normal invasion of the parasites in the first cycle but in the subsequent cycles the merozoites are defective thus producing the abnormal growth patterns observed. It may be speculated that the hybrid glycophorin Dantu relationship

with the integral proteins is such that it impairs the normal utilization of these proteins by the parasite during intraerythrocytic growth. Further work is required to elucidate these mechanisms.

5 APPENDICES

5.1 Acknowledgements

I would like to express my sincere thanks to the following:-

My supervisors, Professor Barry Mendelow and Dr Ernst Hempelmann, whose guidance and encouragement has been invaluable throughout this project.

Maria de Almeida, Sandy Evans and Robyn van Zyl of the Department of Experimental and Clinical Pharmacology for the supply of parasites in culture at often inconvenient times and their sound technical advice.

Professor Felix Monteagudo and Dr Ivan Havlik for allowing me the use of facilities in the Department of Experimental and Clinical Pharmacology.

Professors Alan Fleming and George Nurse for reading and offering constructive criticism of my research protocol.

Dr Phyllis Moores and Mrs Elizabeth Smart of the Natal Blood Transfusion for the supply of S-s-U- and Dantu cells.

Professor Ernette du Toit of the Provincial Laboratory for Tissue Immunology Cape Town for supply of Dantu cells.

Mr Basil van Zyl of SABTS for supply of blood from donors for Henshaw typing.

HBTS for the supply of plasma for use in the cultures.

Mr Mike Lanesman for the excellent photography.

5.2 Raw data obtained from experimental work

5.2.1. Henshaw vs control

Serial Cultures.

	Henshaw				CONTROL			
Hours in Culture	0	24	48	72	0	24	48	72
	0.5	1.8	0.9	3.6	0.9	1.9	2.0	6.1
	-	2.1	2.5	7.1	-	2.1	2.5	6.1
	-	1.4	0.9	5.7	-	1.9	1.6	7.0
	-	1.9	1.2	6.1	-	1.9	2.3	7.9
	-	0.3	1.3	4.2	-	1.5	1.9	2.6
	-	1.0	0.6	3.1	-	1.5	1.7	3.5
	-	2.1	1.9	4.7	-	1.5	1.6	5.7
	-	1.1	0.8	3.8	-	1.1	1.6	7.1
	-	2.1	2.7	8.3	-	1.3	1.0	5.1
	-	0.8	1.5	5.4	-	1.0	1.2	4.5
	Relative Parasitaemia							
	1.00	2.00	1.00	3.94	1.00	2.11	2.22	6.72
	1.00	2.33	2.78	7.83	1.00	2.33	2.78	6.72
	1.00	1.56	1.00	6.28	1.00	2.11	1.78	7.72
	1.00	2.11	1.33	6.72	1.00	2.11	2.56	8.72
	1.00	0.33	1.44	4.61	1.00	1.67	2.11	2.83
	1.00	1.11	0.67	3.39	1.00	1.67	1.89	3.83
	1.00	2.33	2.11	5.17	1.00	1.67	1.78	6.28
	1.00	1.22	0.89	4.17	1.00	1.22	1.78	7.83
	1.00	2.33	3.00	9.17	1.00	1.44	1.11	5.61
	1.00	0.89	1.67	6.06	1.00	1.11	1.33	4.94
Mean	1.00	1.62	1.59	5.73	1.00	1.74	1.93	6.12
SEM	0.00	0.22	0.25	0.58	0.00	0.13	0.16	0.58

	Henshaw				CONTROL			
Hours in Culture	0	24	48	72	0	24	48	72
	2.20	8.93	9.17	14.62	2.40	8.93	8.67	16.02
		6.83	5.77	12.32		7.03	5.97	12.42
		6.73	5.77	9.02		9.73	8.27	13.02
		8.53	8.37	13.02		7.53	5.97	12.62
		6.93	6.47	11.62		9.53	9.17	13.92
		6.53	6.27	10.42		8.73	8.77	16.52
		6.83	5.37	9.02		6.73	5.97	13.72
		7.33	5.67	10.02		6.93	6.27	12.42
		7.03	6.77	11.02		9.53	8.37	15.72
		6.93	7.27	11.12		6.93	5.67	10.72
	Relative Parasitaemia							
	1.00	4.06	4.17	6.64	1.00	3.72	3.61	6.67
	1.00	3.11	2.62	5.60	1.00	2.93	2.49	5.17
	1.00	3.06	2.62	4.10	1.00	4.06	3.44	5.42
	1.00	3.88	3.80	5.92	1.00	3.14	2.49	5.26
	1.00	3.15	2.94	5.28	1.00	3.97	3.82	5.80
	1.00	2.97	2.85	4.73	1.00	3.64	3.65	6.88
	1.00	3.11	2.44	4.10	1.00	2.81	2.49	5.72
	1.00	3.33	2.58	4.55	1.00	2.89	2.61	5.17
	1.00	3.20	3.08	5.01	1.00	3.97	3.49	6.55
	1.00	3.15	3.30	5.05	1.00	2.89	2.36	4.47
Means	1.00	3.30	3.04	5.10	1.00	3.40	3.04	5.71
SEM	0.00	0.12	0.18	0.25	0.00	0.16	0.19	0.25

Combined results

	Henshaw				CONTROL			
Hours in Culture	0	24	48	72	0	24	48	72
Mean 1	1.00	1.83	1.79	6.45	1.00	1.74	1.93	6.12
Mean 2	1.00	3.30	3.04	5.10	1.00	3.40	3.04	5.71
Overall Means	1.00	2.56	2.42	5.78	1.00	2.57	2.48	5.92
SEM		0.74	0.63	0.68		0.83	0.55	0.21

5.2.2. S-s-U- vs control

	S-s-U-				CONTROL			
Hours in Culture	0	24	48	72	0	24	48	72
	0.9	0.5	1.1	1.9	0.2	0.7	1.3	2.7
	0.3	0.7	1	2.4				
	0.7	0.4	0.9	1.8				
	0.1	0.3	0.8	2.1				
	Relative Parasitaemia							
	1	0.56	1.22	2.11	1.00	3.50	6.50	13.5
	1	0.78	1.11	2.67				
	1	0.44	1.00	2.00				
	1	0.33	0.89	2.33				
Mean	1.00	0.53	1.06	2.28	1.00	3.50	6.50	13.50

	S-s-U-				CONTROL			
Hours in Culture	0	24	48	72	0	24	48	72
	3.0	7.2	5.8	7.2	2.4	8.9	8.7	12.2
	-	9.3	8.5	7.9		7.0	6.0	9.6
	-	6.9	5.8	9.9		9.7	8.3	8.6
	-	6.5	5.9	11.2		7.5	6.0	9.6
	-	6.8	5.6	9.2		9.5	9.2	9.6
	-	6.7	6.2	7.2		8.7	8.8	12.0
	-	9.0	7.8	6.9		6.7	6.0	10.0
	-	6.9	5.3	6.8		6.9	6.3	9.2
	-	6.4	6.1	11.0		9.5	8.4	11.4
	-	8.6	8.3	14.5		6.9	5.7	7.2
	Relative Parasitaemia							
	1.00	2.40	1.93	2.40	1.00	3.72	3.61	6.67
	1.00	3.10	2.83	2.63	1.00	2.93	2.49	5.17
	1.00	2.30	1.93	3.30	1.00	4.06	3.44	5.42
	1.00	2.17	1.97	3.73	1.00	3.14	2.49	5.26
	1.00	2.27	1.87	3.07	1.00	3.97	3.82	5.80
	1.00	2.23	2.07	2.40	1.00	3.64	3.65	6.88
	1.00	3.00	2.60	2.30	1.00	2.81	2.49	5.72
	1.00	2.30	1.77	2.27	1.00	2.89	2.61	5.17
	1.00	2.13	2.03	3.67	1.00	3.97	3.49	6.55
	1.00	2.87	2.77	4.83	1.00	2.89	2.36	4.47
Mean	1.00	2.48	2.18	3.06	1.00	3.40	3.04	5.71
SEM	0.00	0.12	0.13	0.26	0.00	0.16	0.19	0.25

Combined data.

	S-s-U-				CONTROL			
Mean 1	1.00	0.53	1.06	2.28	1.00	3.50	6.50	13.50
Mean 2	1.00	2.48	2.18	3.06	1.00	3.40	3.04	5.71
Overall Mean	1.00	1.51	1.62	2.67	1.00	3.45	4.77	9.60
SEM	0.00	0.97	0.56	0.39	0.00	0.05	1.73	3.90

5.2.3. Dantu vs control

	Dantu				CONTROL			
Hours in Culture	0	24	48	72	0	24	48	72
	0.5	1.0	0.9	1.8	0.8	1.5	1.6	5.7
	-	0.9	1.1	1.9	-	1.4	1.5	5.7
	-	1.0	1.2	1.8	-	1.4	1.7	5.0
	-	0.8	1.1	2.0	-	1.6	1.7	5.4
	-	0.9	1.1	1.8	-	1.2	1.4	4.4
	-	1.0	1.0	1.8	-	1.4	1.6	6.5
	Relative parasitaemia							
	0	24	48	72	0	24	48	72
	1.00	1.94	1.75	3.38	1.00	1.92	2.04	7.13
	1.00	1.75	2.13	3.56	1.00	1.79	1.92	7.13
	1.00	1.94	2.31	3.38	1.00	1.79	2.17	6.25
	1.00	1.56	2.13	3.75	1.00	2.04	2.17	6.75
	1.00	1.75	2.13	3.38	1.00	1.54	1.79	5.50
	1.00	1.94	1.94	3.38	1.00	1.79	2.04	8.13
Mean	1.00	1.81	2.06	3.47	1.00	1.81	2.02	6.81
SEM	-	0.06	0.08	0.06	0.00	0.07	0.06	0.36

	Dantu				CONTROL			
Hours in Culture	0	24	48	72	0	24	48	72
	0.37	0.77	0.43	1.63	0.17	0.60	0.50	3.40
	0.37	0.57	0.53	1.53	0.17	0.60	0.50	3.20
	0.37	0.67	0.43	1.43	0.17	0.60	0.40	3.10
	0.37	0.47	0.43	1.53	0.17	0.60	0.60	3.20
	0.37	0.47	0.43	1.63	0.17	0.50	0.50	3.00
	0.37	0.47	0.43	1.93	0.17	0.40	0.60	3.20
	Relative Parasitaemia							
	1.00	2.08	1.16	4.41	1.00	3.53	2.94	20.00
	1.00	1.54	1.43	4.14	1.00	3.53	2.94	18.82
	1.00	1.81	1.16	3.86	1.00	3.53	2.35	18.24
	1.00	1.27	1.16	4.14	1.00	3.53	3.53	18.82
	1.00	1.27	1.16	4.41	1.00	2.94	2.94	17.65
	1.00	1.27	1.16	5.22	1.00	2.35	3.53	18.82
Mean	1.00	1.54	1.21	4.36	1.00	3.24	3.04	18.73
SEM	0.00	0.14	0.05	0.19	0.00	0.20	0.18	0.32

	Dantu				CONTROL			
Hours in Culture	0	24	48	72	0	24	48	72
	0.4	0.6	1.6	2.3	0.2	0.6	0.9	2.7
	0.4	0.9	1.6	2.3	0.2	0.7	1.2	2.6
	Relative Parasitaemia							
	1.00	1.50	4.00	5.75	1.00	3.00	4.50	13.50
	1.00	2.25	4.00	5.75	1.00	3.50	6.00	13.00
Mean	1.00	1.88	4.00	5.75	1.00	3.25	5.25	13.25
SEM	-	0.5	0	0	-	0.25	0.75	0.25

	Dantu				CONTROL			
Hours in Culture	0	24	48	72	0	24	48	72
Mean 1	1.00	1.81	2.06	3.47	1.00	1.81	2.02	6.81
Mean 2	1.00	1.54	1.21	4.36	1.00	3.24	3.04	18.73
Mean 3	1.00	1.88	4.00	5.75	1.00	3.25	5.25	13.25
Overall mean	1.00	1.74	2.42	4.53	1.00	2.77	3.44	12.93
SEM	0.00	0.10	0.83	0.66	0.00	0.48	0.95	3.44

5.3 Abbreviations used in the text

ACD	Acid Citrate Dextrose
C-Terminus	Carboxyl end of peptide
cDNA	Complimentary deoxyribose nucleic acid
CV	Coefficient of Variation
dC	deoxycytidine
dG	deoxyguanosine
EBA-175	Erythrocyte Binding Antigen 175 Kilodaltons.
Fy^a	Duffy blood group antigen a
Fy^b	Duffy blood group antigen b
Ge	Gerbich
GP	Glycophorin
HBTS	Highveld Blood Transfusion Service
He	Henshaw
Hu	Hunter
kDa	Kilodaltons
Mi	Miltenberger
N-terminus	Amino end of peptide acid
p	probability
PBS	Phosphate Buffered Saline
pD protein	Fraction of Duffy blood group antigen
PkDAP-1	*P.knowlesi* Duffy associated protein
PvDAP-1	*P.vivax* Duffy associated protein
SABTS	South African Blood Transfusion Service
SDS PAGE	Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis

SD	Standard Deviation
SEM	Standard error of the mean
SGP	Sialoglycoprotein
St^a	Stones antigen
t	Student's t
T_M	melting temperature
Wr(b)	Wright b antigen

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Author: Field S.P
Name of thesis: Blood group polymorphisms in Southern Africa and innate resistance to plasmodium falciparum

PUBLISHER:
University of the Witwatersrand, Johannesburg
©2015

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