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

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

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

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

MATERIALS AND METHODS

Collection of blood samples

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

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

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

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

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

Invasion of reticulocytes

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

Cell separation by age using centrifugation

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

Intracellular distribution of HbF and malarial parasites

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

Infection of cells with P. falciparum in vitro

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

Growth measurements

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

RESULTS

Invasion of red cells containing HbF

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

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

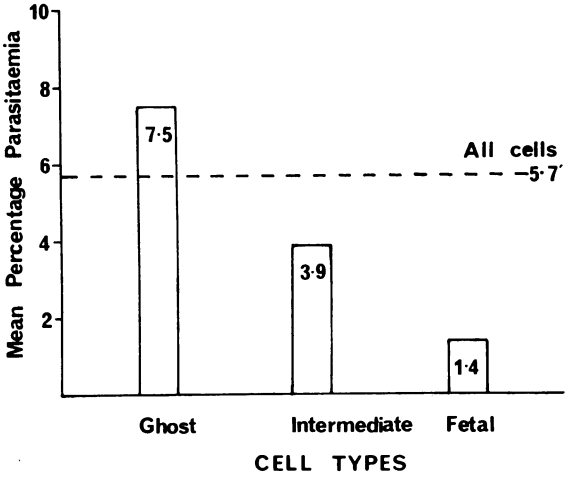

Fig. 1. The distribution of parasites in erythrocytes containing HbA (ghost) or HbF, in blood smears from 13 infants infected with *P. falciparum*.

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

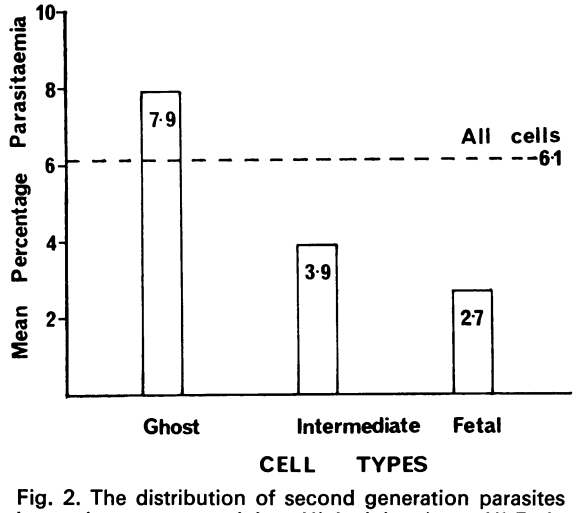

Fig. 2. The distribution of second generation parasites in erythrocytes containing HbA (ghost) or HbF, in tissue cultures of blood from four infants infected with *P. falciparum*.

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

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

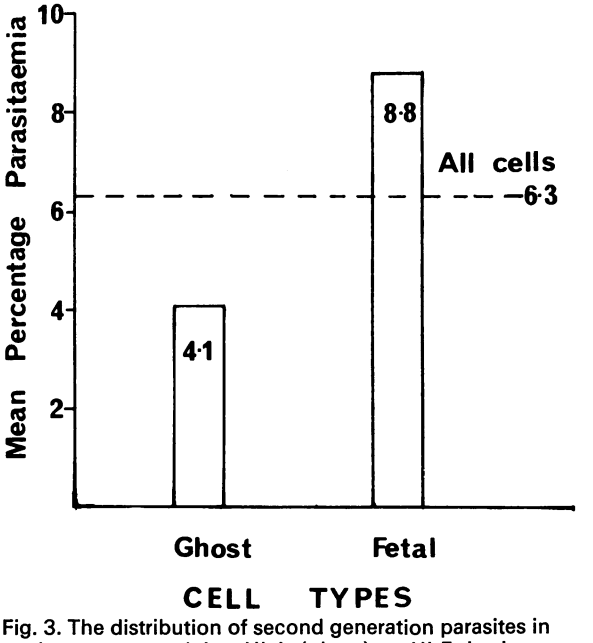

Fig. 3. The distribution of second generation parasites in erythrocytes containing HbA (ghost) or HbF, in tissue cultures in which infected blood (HbA) was mixed with an equal volume of uninfected umbilical cord blood (mostly HbF).

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

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

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

Cell age and susceptibility to infection

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

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

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

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

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

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

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

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

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

Effect of HbF on the growth of intra-erythrocytic parasites

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

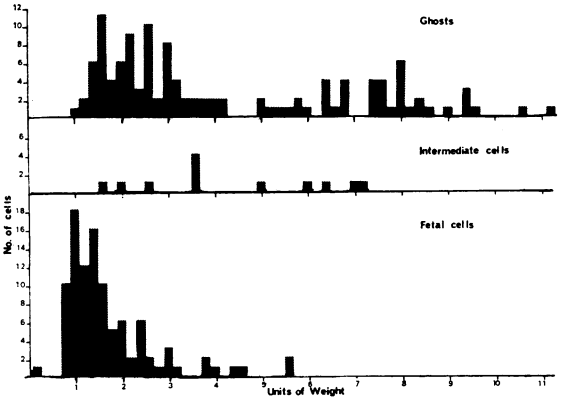

Fig. 4. The 'size' distribution of individual second generation parasites growing in tissue cultures in singly-infected erythrocytes that contained either HbA (119 instances), HbF (100 instances) or an intermediate mixture of HbA and HbF (12 instances). 'Size' measurements were obtained from weighed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

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

DISCUSSION

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

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

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

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

Interaction of falciparum malarial parasites with membrane components that disappear during red cell aging might explain the preferential invasion of erythrocytes. Profound surface reorganization occurs in young erythrocytes during transition from the reticulocyte; surface antigens such as HL-A disappear at this time (*12*). Other studies have shown that old erythrocytes contain 30% less D-galactose residues on their surface than young cells (*13*). The relative resistance of " older " erythrocytes to invasion by *P. falciparum* might provide a temporary protective mechanism against malarial infection in newborn infants. After birth, erythropoiesis shuts off completely and for about a month existing red cells are steadily lost from the circulation as they age. Replacement with new red cells does not occur until haemoglobin levels physiological for the newborn are reached (*3*). The reduced numbers of young, metabolically active red cells in the circulation at this time would confer some degree of protection against fulminating malarial parasitaemias.

In our study, the peripheral blood smears of a few infected infants (5 out of 19) were exceptional in that little difference was seen in the distribution of parasites between cells containing HbA and those containing HbF. This might have resulted from the production for a more prolonged period than usual of red cells that contained HbF. Alternatively, it might indicate the existence of parasites with different invasive capabilities (*14*).

In the investigations of Raper (*15*) and Luzatto (*11*, *16*) on the interaction between falciparum malarial parasites and red cell genetic variants containing HbS or glucose-6-phosphate dehydrogenase deficiency, three possible mechanisms of intrinsic cellular resistance were considered: (1) " failure of infection " due to discrimination at the red cell surface membrane; (2) " abortive infection " due to poor intracellular growth of the parasite; and (3) " suicidal infection " due to the destruction of infected erythrocytes before completion of the parasite's intracellular development. Our investigations have demonstrated that the aging of red cells increases their resistance to infection (mechanism 1). It is perhaps more important that we have strengthened our earlier data (*5*), which suggested that the growth of *P. falciparum* is retarded in cells containing HbF (mechanism 2). The observations illustrated in Fig. 4 were made from parasites growing in fetal cells from umbilical cord blood. In further unpublished work (Pasvol et al., in preparation), we have extended this finding to HbF-containing erythrocytes in the blood of infected infants and subjects with β-thalassaemia or HPFH. Pigment granules are produced in the course of the growth of stunted parasites in HbF-containing erythrocytes, but the way in which HbF exerts its deleterious effect on the parasite is not yet understood. Weatherall (*17*) has suggested that the high affinity of HbF for oxygen might render the parasites relatively hypoxic.

As HbF production persists for longer than usual in the first years of life in infants with thalassaemia and some of the other congenital haemolytic anaemias (*4*), the antiparasitic (growth) effect we have associated with HbF might confer a selective advantage on such children during their early childhood years in malarious areas until protective immunity is acquired. Such an advantage might have helped to maintain the high gene frequencies associated with these disorders in areas where malaria is or has in the past been prevalent.

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RÉSUMÉ

INVASION ET CROISSANCE DE *PLASMODIUM FALCIPARUM* DANS DIFFÉRENTS TYPES D'ÉRYTHROCYTES HUMAINS

Les auteurs ont étudié en cultures tissulaires la sensibilité des érythrocytes humains à l'invasion par *Plasmodium falciparum*, sur différentes populations d'érythrocytes contenant l'hémoglobine fœtale (HbF). Ils ont observé une invasion préférentielle des érythrocytes contenant HbF dans le sang du cordon ombilical. Les parasites n'ont pas manifesté de préférence pour les cellules HbF du sang d'un sujet présentant héréditairement une persistance de l'hémoglobine fœtale (HPFH). En revanche, on a noté une importante préférence pour les érythrocytes HbA du sang des jeunes enfants.

D'autres expériences ont montré que les parasites falciparum envahissaient de préférence les érythrocytes « jeunes ». Ceci pourrait expliquer les répartitions parasitaires observées dans le sang HbF puisque les érythrocytes HbF sont « plus jeunes » en moyenne dans le sang du cordon ombilical, « plus vieux » dans le sang des jeunes enfants, et du même âge moyen que les cellules HbA dans l'HPFH. Les auteurs en ont conclu que la sensibilité des érythrocytes humains à l'invasion par *Plasmodium falciparum* était sans corrélation avec la présence des HbF, mais que le vieillissement des érythrocytes *in vivo* diminuait leur sensibilité à l'invasion.

On a effectué des mesures semi-quantitatives de la croissance des parasites dans les cellules contenant HbA ou HbF. Dans ces dernières, elle était notablement retardée. Ce fait pourrait conférer un avantage sélectif aux individus atteints d'une anémie hémolytique telle que la thalassémie et la drépanocytose, où les taux d'HbF sont augmentés.

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