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Erythrocytic mechanism of sickle cell resistance to malaria

(*Plasmodium falciparum*/hemoglobin S/*in vitro* culture/cyanate)

MILTON J. FRIEDMAN

The Rockefeller University, New York, New York 10021

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ABSTRACT **The physiological basis for the resistance to falciparum malaria of individuals with sickle cell trait has not been understood. Recent advances in erythrocytic *Plasmodium falciparum* culture have made possible a direct investigation of the development of the malaria parasite in cells with sickle cell hemoglobin. In a high (18%) oxygen atmosphere, there is no apparent sickling of cells, and the growth and multiplication of *P. falciparum* is identical in normal (AA), hemoglobin S homozygous (SS), and hemoglobin S heterozygous (SA) erythrocytes. Cultures under low (1–5%) oxygen, however, showed clear inhibition of growth. The sickling of SS red cells killed and lysed most or all of the intracellular parasites. Parasites in SA red cells were killed primarily at the large ring stage, probably as a result of a disruption of the parasite metabolism. Incubation in cyanate prior to culture reversed the resistance of SA erythrocytes to plasmodium growth, but had no effect on SS red cell sickling or resistance. Thus, the mechanism of resistance *in vivo* may be due solely to intraerythrocytic conditions.**

In 1949, the elucidation of the genetic basis for sickle cell anemia (SS) and sickle cell trait (SA) (1, 2) and the demonstration of the involvement of an abnormal hemoglobin in the disease (3) formed a link between genes and molecules. At the same time, medical scientists working in Africa suggested that sickle cell gene carriers were resistant to falciparum malaria (4, 5), thereby forging a connection between molecules and selective pressure. It was proposed that the inheritance of this variant hemoglobin gene conferred resistance in the heterozygous state to falciparum malaria, which is often lethal in the early years of life. The greater ability of heterozygotes to survive to the age of reproduction was thought to balance the almost complete mortality of the homozygous state. In the entire chain between genes and evolution, the weakest link has been that describing the intimate relationship between sickle cell hemoglobin (Hb S) and the malaria parasite, *Plasmodium falciparum*. Population studies have confirmed that children heterozygous for Hb S are protected, statistically, from death, though not infection, by *P. falciparum* (6). Thus far, however, a direct connection between sickling and resistance has remained suggestive and speculative.

The erythrocytic cycle of the parasite proceeds from an infective form, the merozoite. Infection occurs by induced endocytosis, and the growth of the early ring to a trophozoite follows. Nuclear division precedes segmentation of the mature schizont into 16 or more merozoites, which are released upon lysis of the red blood cell. The entire cycle requires 48 hr, the latter two-thirds with the parasitized cell sequestered in the vascular network of the tissues. The periodic fever of malaria results from the synchronous lysis of infected cells upon maturation of the parasite.

In the Hb S-containing red cell, the parasite may not find as perfect a host as the one to which it is adapted. The highly specific needs of *P. falciparum* are reflected in its narrow host range. The only cells known to support the growth of its blood stages are erythrocytes of human beings and a few other primates (7). Thus, any deviation from the normal human red cell might result in an inhibition of infection or growth. Upon deoxygenation, Hb S forms paracrystalline needles which distort both SS and SA cells into their characteristic sickle shape and alter the intracellular viscosity (8). In addition, membrane components (9) and the concentrations of metabolic intermediates (10) are abnormal in the SS cell. A partial inhibition of only one parasite function in SA and SS cells could account for the decrease in severity of the disease seen in the sickle cell gene carrier.

Previously, it was only possible to study a few hours of parasite growth in Hb S-containing red cells. Under these conditions, there was no difference in growth or infection in normal (AA) or SA cells (11, 12). The recently developed continuous culture system for *P. falciparum* in human erythrocytes (13) allows a more complete study of growth in these and other variant cells. This paper describes the conditions under which Hb S-containing red cells become refractory for malaria parasite growth.

MATERIALS AND METHODS

The Parasite and Its Host. A strain of *P. falciparum* (FMG/FCR-3) isolated in West Africa (14) was maintained in normal human erythrocytes (New York Blood Center, type A+), and was used in all experiments. Red cells with abnormal Hbs were supplied through the generosity of E. Roth and R. Nagel of Albert Einstein College of Medicine, New York. Hemoglobin contents were verified by electrophoresis on cellulose acetate at pH 8.6. All red cells were stored in citrate/phosphate/dextrose for up to 4 weeks at 4°. Before use they were washed twice in RPMI 1640 (GIBCO) plus 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate (Hepes) (Sigma Chemical Co.) and resuspended in the culture medium, RPMI 1640 plus 25 mM Hepes with 10% human serum (New York Blood Center, type AB).

The basic technique of growing *P. falciparum* in settled layers of red cells in petri dishes under an atmosphere of 18% O_2/3% CO_2 has been described (15). Modifications of that technique are as follows: All initial cell suspensions were 6% to allow parasite growth unhindered by a thick cell layer. Gentamicin sulfate (Schering Pharmaceutical Corp.) was added to a final concentration of 40 μg/ml. Culture vessels varied but were always flat-bottomed and received 0.2 ml of cell suspension per cm^2 of surface area. High (18%) O_2 atmospheres were established by a burning candle in a glass desiccator as de-

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Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate.

scribed; other atmospheres were prepared by adding a defined gas mixture until outflow was identical to inflow. Cultures were generally asynchronous.

Gas Analysis. The Instrumentation Laboratory 213 blood gas system was used to monitor gas composition before and after 24-hr incubations. All calibrations were to commercial standards and air at 37°. Gas compositions are expressed as percent of total volume.

Microscopic Analysis. Smears of all cultures were made daily and stained with Giemsa's stain. Differential counts of parasite stages roughly correspond to the first (ring), second (trophozoite), and last (schizont) third of the erythrocytic life cycle.

When necessary to preserve red cell morphology, cultures were fixed by adding formalin to 10% through a rubber septum in the lid of the culture vessel.

RESULTS

P. falciparum cultures in Hb S-containing erythrocytes were initiated by inoculation with parasitized AA cells resulting in at least a 30-fold dilution of the inoculum. The blood cells from 11 SS donors (3 with 10–20% Hb F), 4 SA donors, and 20 AA donors were tested. Under an atmosphere of high (18%) oxygen, in which there was no sickling, there was no difference in the extent of parasite multiplication after 4 days. Thus, none of the cell types studied was resistant to parasite infection or growth. Parasite morphology was in all cases normal, and infected variant erythrocytes looked like normal infected cells.

When the oxygen tension was lowered, however, there were substantial differences among these types of blood. Because the *P. falciparum* erythrocytic cycle is 48 hr *in vivo* and *in vitro*, 2 days of growth at high oxygen is necessary and sufficient to establish the infection in variant red cells. At this time, single 1.6-ml cultures were split into 0.4-ml cultures and incubated at high and low oxygen tensions in parallel (Fig. 1). After 24 hr under a 3% O_2/3% CO_2/94% N_2 atmosphere, 96% of the red cells in the SS culture were sickled. There were few or no parasites visible within these cells. Under the same atmosphere, approximately 60% of the SA cells were sickled or distorted. The number of parasites had increased, but some appeared abnormal (Fig. 2). After another day at low oxygen, there were very few parasites with normal morphology in SA red cells. Most were condensed and clearly dead. Multiplication in AA cells was normal, and the parasites had normal morphology.

Growth curves in the three types of cells at four different O_2 tensions are shown in Fig. 3. These oxygen tensions resemble common (5%) and extreme (1%) physiological conditions in venous circulation. The pH of the cultures ranged from 7.4 at the start to 7.2–7.0 after 24 hr.

AA and SS cells were only slightly affected by variations in oxygen between 1 and 5%, but SA cells were affected more, both in their extent of sickling and in their resistance to parasite growth. The correlation between these phenomena extended to variations between individuals of the same genotype. That is, SA blood that showed a greater tendency to sickle proved to be a less hospitable host, though these differences were minor compared to those seen between red cell variants.

A differential count was made of those parasites surviving the first day of low oxygen culture in SA red cells. These data are given in Table 1 with counts of parasites in AA red cells and those in SA red cells at high O_2. In SA red cells, the development of the rings to larger forms was specifically inhibited at low O_2. After one day (day 3), there were 9.6 trophozoites and schizonts per 1000 red cells at 18% O_2, while there were only 0.7 at 3%

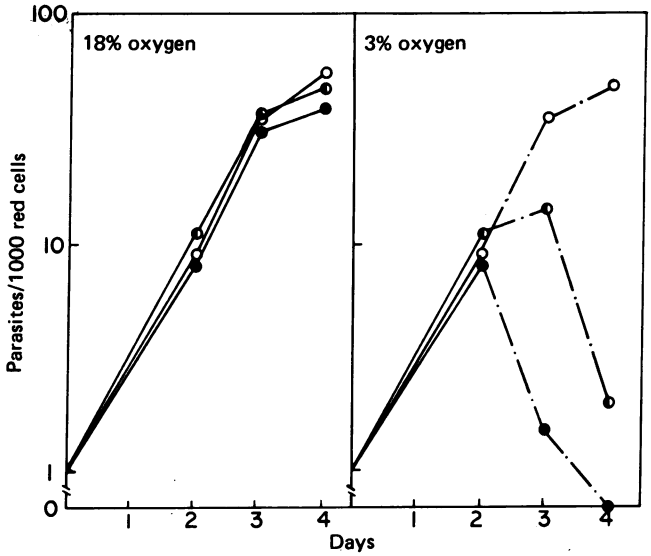

FIG. 1. Growth of *P. falciparum* in Hb S-containing erythrocytes. Cultures were grown under an atmosphere of 18% O_2/3% CO_2 for 2 days and then either maintained in this gas mixture (*Left*) or shifted to 3% O_2/3% CO_2 (*Right*). Data shown are typical of growth in AA (○), SA (◐), and SS (●) red cells.

O_2, an inhibition of over 90%. In contrast, the schizonts present on day 2 were able to develop, and, furthermore, the resultant merozoites were able to invade new hosts to a significant extent. Since no class of parasites survived 24 hr of low O_2 growth in SS cells, one can reason that at least half of the cell cycle is susceptible to destruction in these cultures.

The carbamylation of Hb S with cyanate leads to an inhibition of sickling (16), primarily by increasing the oxygen affinity of the hemoglobin (17). SS, SA, and AA red cells were incubated for 1 hr at 37° in RPMI-1640 plus 25 mM Hepes without serum and with 0, 5, or 50 mM NaNCO. They were then washed four times in 20 volumes of RPMI-1640 plus 25 mM Hepes, suspended in medium with 10% serum, and inoculated as above. The sickling of SS red cells and the resistance of these cells to *P. falciparum* were not affected by cyanate under these conditions (Table 2). SA cells, however, showed a decrease in the percent of sickling and a significant reversal of protection against parasite growth.

DISCUSSION

The resistance of carriers of the sickle cell gene to malaria can be linked directly to a single abnormal protein. Under *in vitro* conditions in which this variant hemoglobin behaves much like the normal variant, there is no inhibition of parasite growth and multiplication. Under deoxygenating conditions, which affect directly and profoundly the physical nature of Hb S, the parasites are severely injured. At the same time, cyanate, an agent that functions exclusively to limit the abnormal functioning of this variant, protects, within those limits, the parasites from destruction. Thus, it can be concluded that the reversible physical state of Hb S mediates the resistance observed *in vitro* and that a solely erythrocytic mechanism is sufficient for resistance *in vivo*. While other mechanisms involving nonerythrocytic cells might in fact operate, they do not appear to be necessary.

Morphological evidence allows one to hypothesize that two mechanisms of killing operate *in vitro*. The complete disappearance of parasites from cultures of SS erythrocytes indicates a lytic process. This might originate from physical disruption

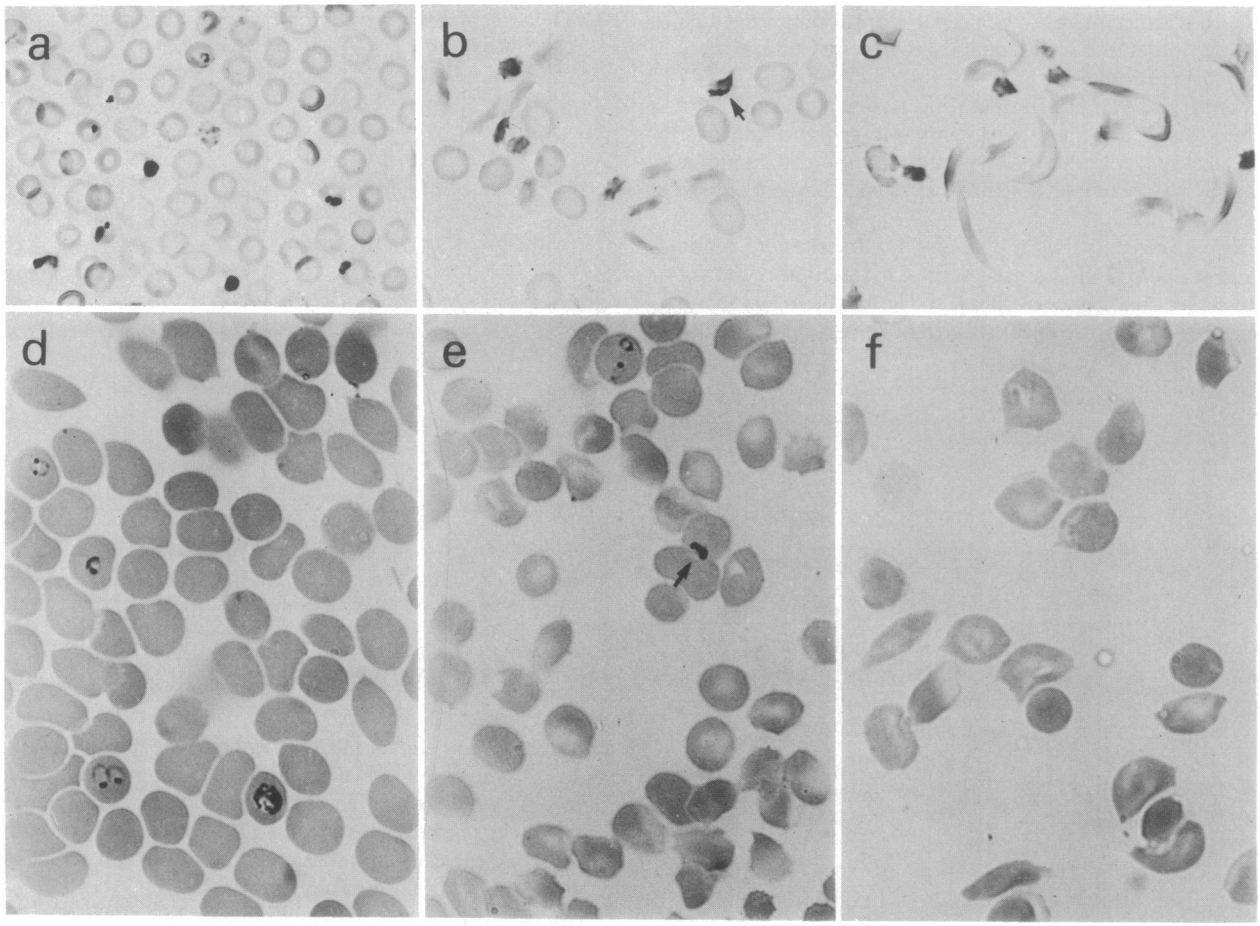

FIG. 2. Photomicrographs of cells from culture after 2 days at 18% O_2 followed by 1 day at 3% O_2. AA (*a*), SA (*b*), and SS (*c*) cells were fixed in 10% formalin, washed, and smeared on slides before they were stained with Giemsa's stain. (×500.) AA (*d*), SA (*e*), and SS (*f*) cells were smeared without prior fixation. (×1000.) The arrow in *b* indicates a sickled, parasitized cell. The arrow in *e* indicates an abnormal parasite form.

of the parasite membranes by paracrystalline Hb S needles and the subsequent liberation of digestive enzymes. Deoxygenated SS red cells have been shown to be densely packed with these Hb S aggregates (18), leaving little room for intracellular parasites. Mechanical disruption, however, is not believed to be the cause of death in SA erythrocytes. At low O_2, rather than being absent, the parasites resemble ones that have been cultured under poor conditions. This suggests a disruption of parasite metabolism in the sickled cell. One possibility is an inhibition of the endocytic feeding process of the plasmodium (19), though a less obvious mechanism may be acting.

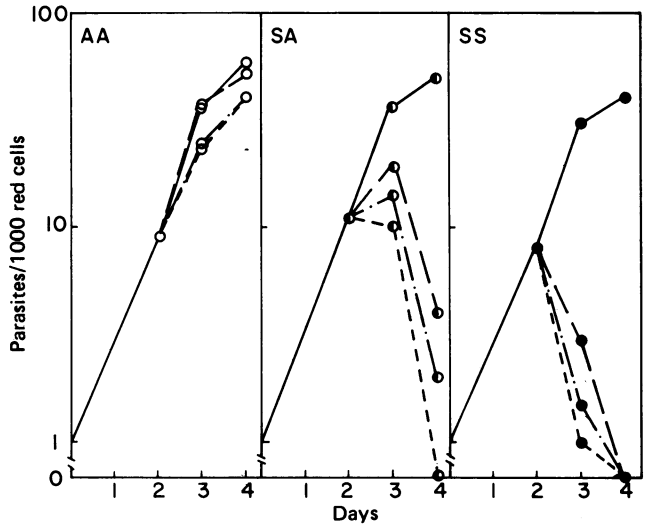

FIG. 3. Effect of variations in oxygen on cultures of *P. falciparum* in AA, SA, and SS erythrocytes. As in Fig. 1, cultures were shifted on day 2 from 18% O_2 to lower oxygen levels. All atmospheres contained 3% CO_2 and 18% (—), 5% (— —), 3% (— · —), or 1% (- - -) oxygen.

This report details only one aspect of the relationship between malaria and the sickle cell, that being the effect of sickling on the *P. falciparum* erythrocytic cycle. The effect of the parasite on sickling should also be considered. Luzzatto *et al.* (20) found preferential sickling of infected erythrocytes when they subjected blood from a malarial SA donor to complete deoxygenation. They suggested that resistance was due to selective sickling and phagocytosis of infected red cells in the spleen.

We have repeated these experiments with SA cells infected *in vitro* (21) and have obtained compatible results with the added observation that schizont-infected cells do not sickle.

Table 1. Differential counts of parasite stages in AA and SA cells

Red cell	Day	% O_2	Parasites/1000 red cells		
			Rings	Trophozoites	Schizonts
AA	2	18	6.3	0.6	0.1
	3	18	25.2	4.2	5.6
	3	3	26.3	5.3	4.3
SA	2	18	5.0	3.1	1.3
	3	18	27.4	4.4	5.2
	3	3	13.3	0.6	0.1

Table 2. Growth of *P. falciparum* in cyanate-treated red blood cells

Red cell	NaNCO, mM	Parasites/1000 red cells 3% O_2	Parasites/1000 red cells 18% O_2	% sickled at 3% O_2
AA	0	26	29	—
	5	27	30	—
	50	26	26	—
SA	0	0	36	60
	5	4	32	56
	50	22	36	43
SS	0	0	48	96
	5	0	44	96
	50	0	41	96

Red cells were treated as described in *Results*. After inoculation, cells were cultured for 2 days at high oxygen and then split as in Fig. 1. After 2 more days, smears were made of both formalin-fixed and unfixed cells. The percent of sickled or distorted cells was determined and the parasites were counted. Parasite counts on day 0 were similar but not equal in this experiment.

(Schizonts in SA cells survive low oxygen culture.) Data presented above show that 90% of young parasites are killed in a population of SA cells of which only 60% are sickled or distorted. This also suggests that the parasite contributes to conditions that induce sickling in its host cell. Lowered intracellular pH and oxygen levels resulting from parasite metabolism may play a role in this effect, as both are known to increase sickling (22).

The mechanism of resistance *in vivo* has yet to be clearly defined. Invasion of SA and SS red cells is not inhibited under 18% oxygen and was not the limiting factor at low O_2 in either of these cell types. Growth, however, is interrupted in both upon sickling of the cells. An unanswered question is whether and where parasitized cells sickle *in vivo*. If they do so in the spleen, it is likely that they are then removed by filtration and finally engulfed by macrophages. If they do not sickle in circulation, they will eventually be sequestered in the capillaries when the parasites become large rings. Depending upon the oxygen concentration and pH, a portion of these cells would sickle and, as seen above, some of the parasites would die. The evidence does not specifically favor either of these mechanisms of resistance. It does allow for two possible models of host–parasite interaction which, together or individually, could cause an attenuation of the rate of parasite multiplication, an attenuation that would allow the immune system time to mount a curative response.

Attempts to measure the resistance of different populations of Hb S heterozygotes to malaria have yielded highly variable results (6). Some of this variability may be related to strain differences of *P. falciparum*. A recent ultrastructural study of three strains has revealed differences in the number and size of infected erythrocyte knobs (S. Langreth, personal communication). These knobs occur on the red cell membrane at the time of sequestration (23) and have been shown to be involved in the attachment of parasitized cells to the endothelial lining of the blood vessels (24). If resistance to malaria is mediated by the location and duration of sequestration, these strain differences could cause the observed variation in protection.

Resistance to malaria in other populations might be mediated by red cell variants as well. Short-term cultures show that red cells containing high levels of hemoglobin F do not support optimal levels of *P. falciparum* growth (25). Thus, the protection in infancy may be due to a variant hemoglobin. Little is known of the interactions between the parasite and other common erythrocyte abnormalities. These include red cells from persons with Hb C disease, glucose-6-phosphate dehydrogenase deficiency, and β-thalessemia, all of which are prevalent in areas endemic for falciparum malaria and all of whose liabilities may be balanced by an inhospitality for the malaria parasite.

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