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Comparison of *Plasmodium falciparum* growth in sickle cells in low oxygen environment and candle-jar

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Abstract

The first successful in vitro cultivation of *Plasmodium falciparum* in sickle cells in a gas mixture containing 3% oxygen, 4% carbon dioxide and 93% nitrogen has been reported recently, contradicting earlier claims that the parasite does not multiply continuously in sickle cell trait (HbAS) and sickle cell anemia (HbSS) erythrocytes at low oxygen tension. The present study extends that report by growing three *P. falciparum* strains in erythrocytes from four different sickle cell trait and four sickle cell anemia donors. Because *P. falciparum* is known to grow normally in sickle cells when incubated in a candle-jar estimated to contain 15–18% oxygen, we have also compared the growth at 3% oxygen with that in a candle-jar. For convenience, we also refer to the 3% oxygen and the candle-jar as low and high oxygen environment, respectively. The three *P. falciparum* strains were first grown continuously in low oxygen environment for at least 1 month in erythrocytes from one HbAS carrier. These stock cultures were then used to infect erythrocytes from additional three HbAS carriers and four HbSS patients. Results of the experiments showed that parasite growth and hemozoin production in HbAS erythrocytes in low oxygen environment were comparable to those obtained in the candle-jar. There was growth retardation in HbSS erythrocytes in low oxygen environment, but some of the parasites survived and eventually produced high parasitemia levels. Continuous cultivation of different *P. falciparum* strains in HbAS erythrocytes is necessary for investigation of possible molecular differences between malaria parasites in sickle cells and those in HbAA erythrocytes.

Keywords: Sickle cells; Malaria; Continuous culture

1. Introduction

Malaria parasites infect 300–500 million people and kill more than a million of them annually (Nahlen et al., 2003), although some investigators have questioned the accuracy of these numbers (Breman, 2001). The numbers obviously include people infected with any of the four *Plasmodium* species (*Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium ovale*) that cause malaria in humans. However, *P. falciparum* infec-

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tions cause most of the malaria deaths (Miller et al., 2002).

About 50 years ago, it was noticed that the incidence of sickle cell trait with HbAS erythrocytes was higher in regions where malaria was prevalent than elsewhere (Allison, 1954). That observation has been repeatedly confirmed over the years and it is now widely accepted that sickle cell trait confers partial protection against severe falciparum malaria (Aidoo et al., 2002). In epidemiological sense, the protection provided by sickle cell trait against death from malaria somehow compensates for the health devastation inflicted by sickle cell disease (Rahimy et al., 2000; Athale and Chintu, 1994). Unfortunately, individuals with sickle cell anemia (HbSS) are not protected from falciparum malaria, perhaps because of their pre-existing poor health (Luzzatto and Pinching, 1990; Neequaye, 1986).

Possible mechanisms of sickle cell trait protection against severe falciparum malaria are still being investigated. It has been suggested that HbAS individuals may have more effective immune responses than normal HbAA individuals have against malaria (Abu-zeid et al., 1992). Other potential mechanisms of sickle cell protection against falciparum malaria include increased HbS polymerization in infected erythrocytes, which may physically damage the parasites (Friedman, 1978) or promote their clearance by phagocytes (Luzzatto et al., 1970), impaired parasite metabolism in HbAS erythrocytes (Friedman, 1978) and retarded parasite growth (Pasvol et al., 1978).

Most of the modern research activities on molecular biology of *P. falciparum* and vaccine development have been using parasites from HbAA erythrocytes only, perhaps due to lack of defined and well-characterized parasites from other erythrocyte types. Large quantities of well-characterized *P. falciparum* strains are usually obtained from in vitro culture, and the preferred method for cultivation of the parasites include incubation in 2–5% oxygen environment. Friedman (1978) has reported that *P. falciparum* growth was severely inhibited in sickle cells at 1–5% partial oxygen tension, although the parasites grew normally in a candle-jar, estimated to contain 18% oxygen. Similarly, Pasvol et al. (1978) have reported decreased invasion of sickle cells by *P. falciparum* and retarded growth at low oxygen atmosphere. However, in 1999, a strain of *P. falciparum* that grew normally and continuously in HbAS erythrocytes in a gas mixture containing 3% oxygen, 4% carbon dioxide and 93% nitrogen was reported (Orjih, 1999). Further studies with that *P. falciparum* strain are presented in the current report together with two additional strains that have also grown continuously in HbAS erythrocytes for several weeks at low oxygen atmosphere.

2. Materials and methods

2.1. Malaria parasites

Three strains of *P. falciparum* were used in the study, including: HB3, PB112 and Amiri7. HB3 was originally cloned from Honduras I/CDC strain in Trager's laboratory (Bhasin and Trager, 1984). PB112 was isolated in Kuwait from an African man whose erythrocytes were sickling test positive. The sickling test was done by mixing a drop of blood with a drop of 0.8% aqueous sodium metabisulfite solution on a glass slide, covering the suspension with a coverslip and sealing it with petroleum jelly. After incubation for 30 min at room temperature, the slide was examined under the microscope for sickled cells (McKenzie, 1996). Hemoglobin electrophoresis was not done because of insufficient blood sample.

P. falciparum Amiri7 strain was isolated from an adult male Indian in Kuwait, whose erythrocytes were sickling test negative. Amiri7 and PB112 were adapted to in vitro growth in our laboratory, and were maintained in HbAA erythrocytes for approximately 2 months before they were used for studies in sickle cells. Other experiments with PB112 strain have already been published (Orjih, 1999).

2.2. Growth medium

RPMI 1640 powder, HEPES, hypoxanthine and gentamicin were obtained from GIBCO BRL (Life Technologies Ltd., Paisley, Scotland). The RPMI medium was prepared essentially according to Jensen and Trager (1977). Each liter was supplemented with 5.95 g of HEPES, 2 g of D-glucose, 50 mg of hypoxanthine and 20 mg of gentamicin. Before use, 10 ml of sterilized human serum and 4.2 ml of 5% sodium bicarbonate solution were added to 85.8 ml of the medium. All sterilizations were done by passing the solution through membrane filters with 0.2 μm pore size, except for the serum which required 0.45 μm pore size.

2.3. Test erythrocytes

The normal HbAA erythrocytes that were used for routine cultivation of the parasites were obtained from Kuwait Central Blood Bank. The HbAS erythrocytes that were used for continuous cultivation of the parasites were collected from a donor who had 63% HbA, 36% HbS and 1% HbF. The remaining sickle cell samples, including blood from three HbAS and four HbSS individuals were supplied by a local hospital. None of the blood donors had a history of blood transfusion later than 6 months before the samples were collected. Of the sickle cell disease donors, one was a female whose medical record showed that she had sickle cell anemia together with beta-thalassemia, one other female had HbSS with high HbF level and the remaining two donors were males who had sickle cell anemia. Detailed hemoglobin analysis could not be carried out because of limited quantities of the blood samples.

All the erythrocytes that were used in this study were tested for sickling in 0.8% sodium metabisulfite solution. Those that were positive were further subjected to sickling test in the gas mixture that contained 3% oxygen, 4% carbon dioxide and 93% nitrogen as is described below.

2.4. Parasite cultures

The parasites were grown essentially by the standard in vitro method (Trager and Jensen, 1976). Unless stated otherwise, the cultures were contained in 50 ml culture flasks (Orjih, 1999). The volume of the culture medium was usually 7 ml and the parasites were normally sub-cultured at intervals of 4–6 days. The cultures were incubated in either low or high oxygen environment.

2.5. Low oxygen environment

Culture containers were placed in a 5-l modular incubator chamber (Billups-Rotthenberg, Del mar, CA, USA). Air in the chamber was displaced completely with gas mixture that contained 3% oxygen, 4% carbon dioxide and 93% nitrogen (Kuwait Oxygen & Acetylene Co., Kuwait), and the gas out- and inlets were sealed airtight.

2.6. High oxygen environment (Candle-jar)

A burning candle was placed in a glass desiccator containing the parasite cultures. The desiccator was covered tightly with the cover, after which the candle fire extinguished by itself within 5 min. This system is called the candle-jar, and it has been estimated to contain 16–18% oxygen (Jensen and Trager, 1977; Scheibel et al., 1979).

2.7. Continuous cultivation of *P. falciparum* in HbAS erythrocytes

The first infection of HbAS erythrocytes was started with *P. falciparum* culture in HbAA erythrocytes. Parasite growth was synchronized at the ring stage by treatment with sorbitol solution (Lambros and Vanderberg, 1979). After synchronization, the parasites were permitted to grow for 30 h before being centrifuged at 3000 rpm for 10 min. The dark film on top of the erythrocyte pellet was carefully removed with a pipette and suspended in a 2 ml of growth medium. Giemsa-stained smears from the suspension showed that most of the parasites were at the trophozoite and schizont stages of growth and the parasitemia was approximately 35%.

The parasite culture was diluted to 0.5% parasitemia with HbAS erythrocytes that were suspended in growth medium. Two 7-ml aliquots of the diluted culture, 6% hematocrit, were prepared in 50-ml flasks; one of them was incubated in low oxygen and the other in a candle-jar. Spent medium in each culture was changed daily, and smears of the cells were prepared, stained with Giemsa stain and examined under the microscope for parasitemia. The parasites were subsequently sub-cultured to fresh HbAS erythrocytes at intervals of 4–6 days, depending on multiplication rate. The three *P. falciparum* strains that were used in this study were similarly adapted to growth in HbAS erythrocytes.

2.8. *P. falciparum* cultivation in HbSS erythrocytes

Washed HbSS erythrocytes were suspended in growth medium at 4.5×10^8 cells per milliliter. *P. falciparum* schizonts in HbAS erythrocytes were mixed with the HbSS erythrocyte suspension to obtain 1% parasitemia. Three milliliter aliquots of the suspen-

sion were prepared in tissue culture plates containing six wells, and then maintained in a low oxygen environment or in a candle-jar with daily change of spent medium. Fresh HbSS erythrocytes were added to the cultures on day 3 to dilute the parasitemia to approximately 0.2% in which most of the parasites were at the trophozoite and schizont stages of growth. The cultures were maintained for 6–8 additional days.

2.9. Sickling effect of low oxygen environment

One milliliter of the parasite culture or uninfected erythrocyte suspension in growth medium was injected with a needle into a vacutainer tube through a rubber stopper. To subject the cells to low oxygen tension, the tube was filled with a mixture of 3% oxygen, 4% carbon dioxide and 93% nitrogen. The gas mixture was introduced into the tube through the rubber stopper with an 18-gauge needle that was attached to the gas tank. The tube was incubated overnight in the modular incubator chamber described above. In some of the experiments with uninfected erythrocytes, the suspension was incubated only for 5 h. After incubation, 0.2 ml of a 25% aqueous solution of glutaraldehyde was injected into the tube through the rubber stopper. The sample was incubated at room temperature for 60 min with occasional mixing. After the fixation procedure, the suspension was centrifuged at 3000 rpm for 10 min. The supernatant was discarded, and the pellet was washed twice with culture medium. Smears of the cells were prepared, air-dried, stained with Giemsa stain and examined under the microscope to evaluate the morphology of the cells.

Hemozoin production was measured quantitatively as has been described previously (Orjih and Fitch, 1993).

3. Results

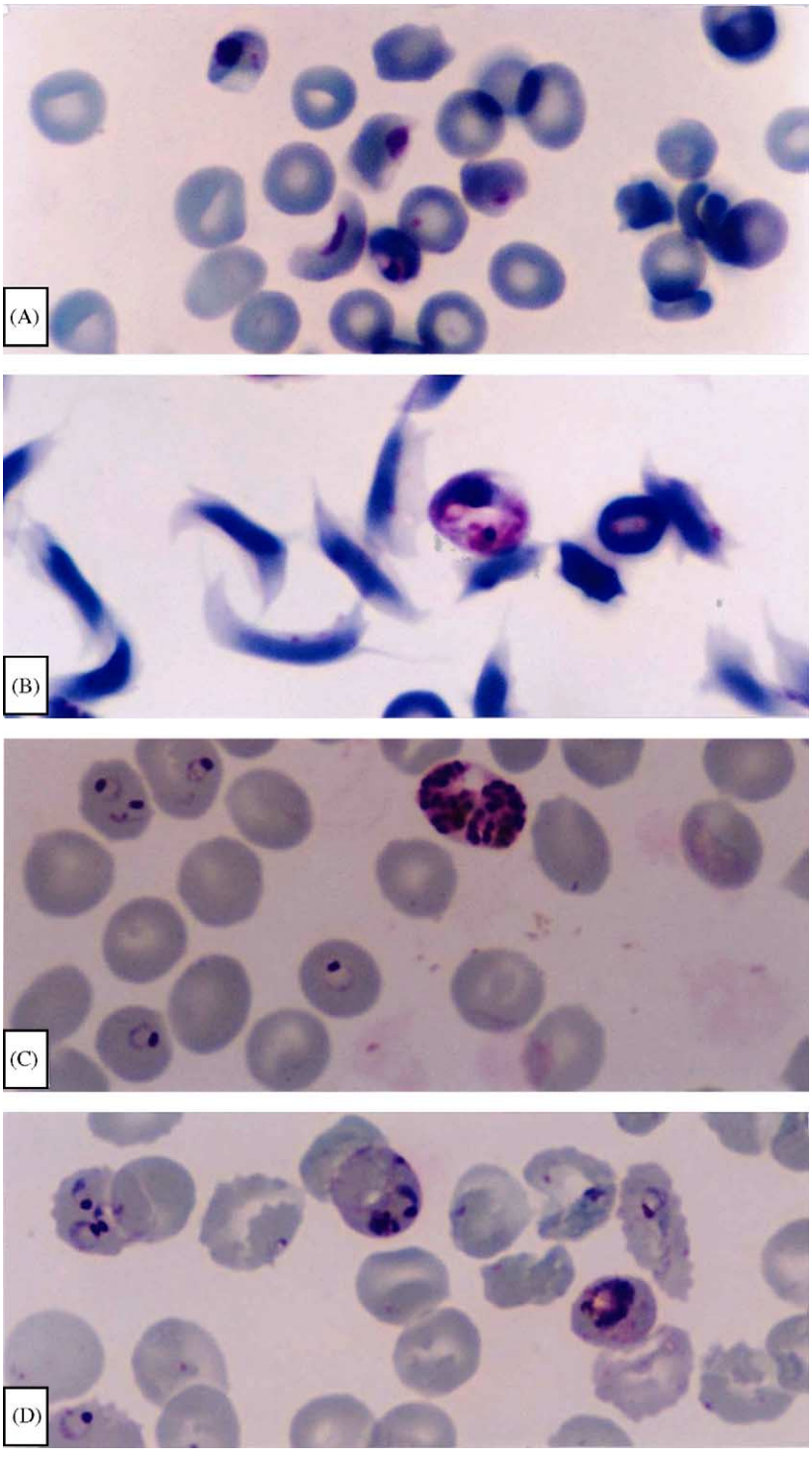
3.1. Effect of low oxygen environment on sickle cells

When parasite cultures in HbAS and HbSS erythrocytes were incubated overnight in the gas mixture containing 3% oxygen, 4% carbon dioxide and 93% nitrogen, and were fixed with glutaraldehyde before exposure to air, approximately 5–9% of HbAS (Fig. 1A) and 84–97% of HbSS erythrocytes (Fig. 1B) sickled. When unparasitized sickle cells were incubated in the gas mixture, it was found that approximately 80–95% of HbSS erythrocytes also sickled. However, less than 2% of the HbAS erythrocytes sickled after overnight incubation in the low oxygen environment, with the sickled cells appearing only somewhat distorted. It was also found that a 5 h exposure to the 3% oxygen gas mixture was sufficient to cause extensive sickling of HbSS erythrocytes, but had little effect on the HbAS erythrocytes (data not presented). A shorter than 5 h exposure time was not tested. These data suggest that the low oxygen gas mixture used in the present study was probably comparable to the low oxygen tension in the deep tissues where *P. falciparum* malaria parasites normally sequester and multiply in humans (Pasvol et al., 1978). Removal of the cell suspension from low oxygen environment without prior fixation with glutaraldehyde allowed sickled erythrocytes to unsickle and appear normal (Fig. 1C and D).

3.2. Growth and multiplication of P. falciparum strains in HbAS erythrocytes

The three *P. falciparum* strains completed the asexual life cycle in all the HbAS erythrocytes within 48 h both in low and high oxygen environments, and the number of merozoites in mature schizonts ranged be-

Fig. 1. In vitro culture of *P. falciparum* in sickle cells in low oxygen environment. The erythrocytes were infected with *P. falciparum* Amiri7 strain and incubated in a gas mixture containing 3% oxygen, 4% carbon dioxide and 93% nitrogen in sealed tubes. Spent medium was replaced with fresh medium every day and the smears shown here were prepared on day 5, when the cells shown in (A) (HbAS) and (B) (HbSS) were fixed with glutaraldehyde before exposure to air to prevent unsickling, whereas the cells shown in (C) (HbAS) and (D) (HbSS) were exposed to air without prior fixation. All the human cells in the photograph are erythrocytes, some of which contain the malaria parasite *P. falciparum*. There are at least six infected erythrocytes in (A) (ring forms and trophozoites), five in (B) (one schizont and four ring forms—small red dots). Parasite morphology was not clearly defined in glutaraldehyde-fixed smears, but the red nuclei were clearly visible under the microscope. The morphology was much more impressive in cultures that were exposed to air without prior fixation. Multiple infections were common in both HbAS and HbSS erythrocytes. Similarly, some multiply-infected schizonts were observed in both HbAS and HbSS cultures. (C) Two mature schizonts in one HbAS erythrocyte and the total number of merozoites in the cell was 22 under the microscope.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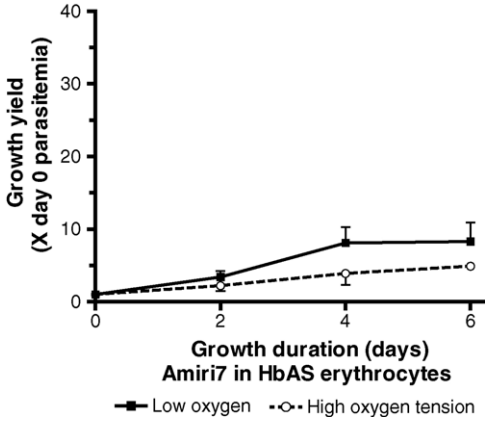

Fig. 2. Comparison of the growth of *P. falciparum* Amiri7 strain in HbAS erythrocytes in low and high oxygen environments. Symbols indicate mean ± standard error of mean of four experiments.

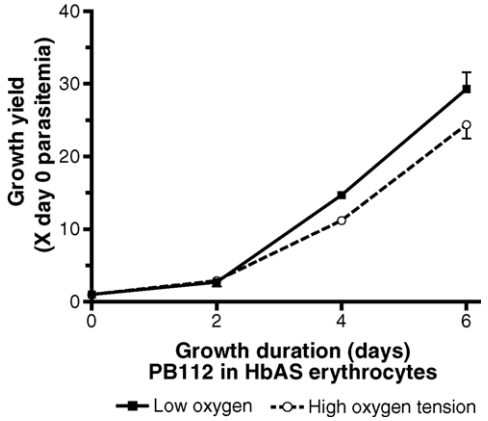

Fig. 4. Comparison of the growth of *P. falciparum* PB112 strain in HbAS erythrocytes in low and high oxygen environments. Symbols indicate mean ± standard error of mean of four experiments.

tween 12 and 20, except in multiply-infected erythrocytes where more than 20 merozoites in a schizont were occasionally observed (Fig. 1C).

Throughout the study period, it was observed that starting the in vitro culture with approximately 0.2–1% parasitemia levels on day 0 usually reached the highest parasitemia level after three asexual multiplication cycles. Figs. 2–4 show the multiplication of the three *P. falciparum* strains from when the parasites were sub-cultured on day 0 until day 6 when they were due to be sub-cultured again. The starting parasitemia was not always the same in all the cultures, and to correct for the differences, the results have been expressed as parasite growth yield, which was calculated through dividing the parasitemia (%) on days 2, 4 and 6 by the parasitemia (%) on day 0 of the experiment. The figures show that, with each of the three strains, parasite multiplication was, on the average, higher in low oxygen environment than it was in the candle-jar. However, the differences between the means were not statistically significant (in *t*-test, the *P*-values ranged from 0.0757 to 0.8625, whereas not higher than 0.05 would have been considered significant).

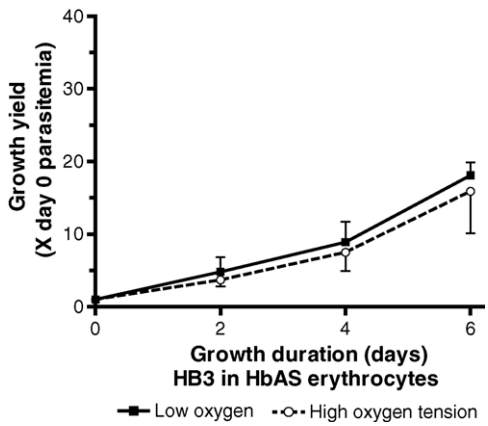

Fig. 3. Comparison of the growth of *P. falciparum* HB3 strain in HbAS erythrocytes in low and high oxygen environments. Symbols indicate mean ± standard error of mean of four experiments.

3.3. Growth and multiplication of *P. falciparum* in HbSS erythrocytes

Because of limited amounts of blood samples, the three *P. falciparum* strains were each tested in erythrocytes from a different HbSS blood donor (see Section 2). During the first 1–2 days in low oxygen environment, *P. falciparum* HB3 growth in HbSS appeared retarded when compared with the same parasite strain in the candle-jar. On day 2, there were about equal proportions of ring forms and schizonts in low oxygen environment, whereas most of the parasites in high oxygen environment had completed the first asexual cycle and were all at the ring stage of the second cycle. On day 6, the parasitemia level was 9× lower in the low than in the high oxygen environment, even though

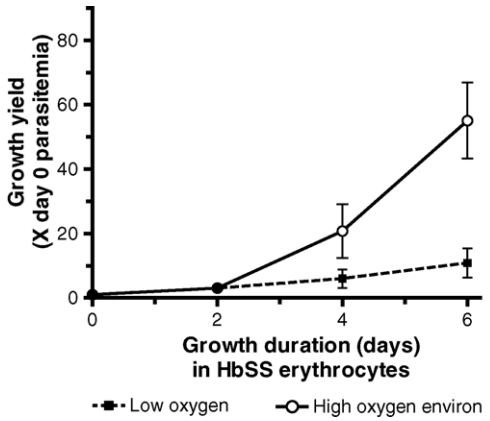

Fig. 5. Growth and multiplication of *P. falciparum* strains in HbSS erythrocytes in low and high oxygen environments. Data obtained with *P. falciparum* Amiri7, HB3 and PB112 strains were pooled and symbols represent the mean ± S.E.M.

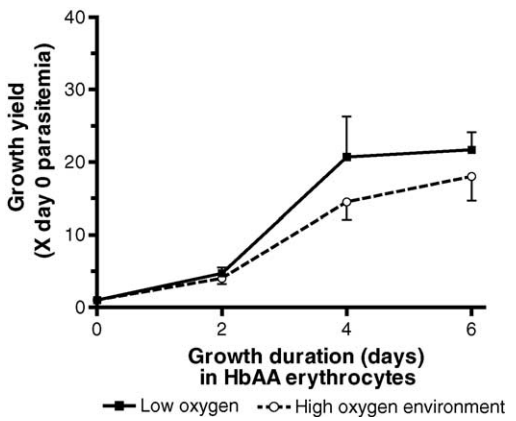

Fig. 6. Growth and multiplication of *P. falciparum* in HbAA erythrocytes in low and high oxygen environments. Results obtained with Amiri7, HB3 and PB112 strains were pooled to obtain the data summarized here. Bars represent mean ± S.E.M. of the three experiments.

the starting parasitemia was identical in both environments.

There was also slight growth retardation of the other two *P. falciparum* strains during the first 24–48 h in HbSS in low oxygen environment, but it was not as striking as with HB3. Fig. 1B and D show *P. falciparum* Amiri7 on day 5 of growth in HbSS erythrocytes in low oxygen environment. The growth yield in each of the three *P. falciparum* strains was higher in the candle-jar than in low oxygen environment (Fig. 5).

3.4. Growth and multiplication of *P. falciparum* in HbAA erythrocytes

In HbAA erythrocytes, the growth and multiplication of the three *P. falciparum* strains were the same as has been described above with parasites in HbAS erythrocytes, both in low and high oxygen environment. The growth yields during a 6-day growth period of synchronized parasites in HbAA erythrocytes have been summarized in Fig. 6.

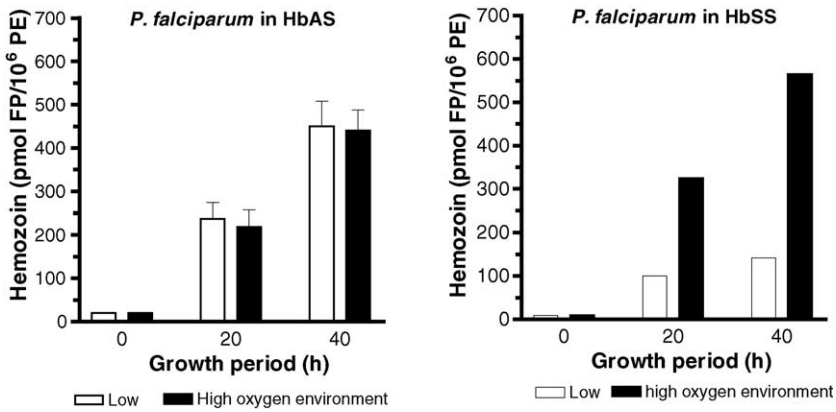

Fig. 7. Hemozoin production by *P. falciparum* in sickle cells. HbAS and HbSS erythrocytes were infected with *P. falciparum* Amiri7 strain and incubated either in low or high oxygen environment. After 4 days, parasite growth was synchronized at the ring stage and the cultures were then incubated for the period indicated in the figure. Experiments with HbAS erythrocytes were done four times with erythrocytes from four different blood donors, but the experiment with HbSS was done once because of limited availability of erythrocytes. PE stands for parasitized erythrocytes.

3.5. Hemozoin production by P. falciparum in sickle cells

Intraerythrocytic production of hemozoin indicates hemoglobin catabolism and ferriprotoporphyrin IX dimerization by malaria parasites (Orjih, 1996). Fig. 7 shows that the quantities of hemozoin that were produced by *P. falciparum* in HbAS erythrocytes during one asexual life cycle remained essentially the same whether the culture was incubated in low or high oxygen environment, indicating that low oxygen environment did not inhibit parasite utilization of host cell hemoglobin. In contrast, hemozoin production in HbSS erythrocytes was clearly inhibited in low oxygen environment, when compared with the production in high oxygen environment.

4. Discussion

The data presented here clearly show that parasite growth and multiplication in HbAS erythrocytes in low oxygen environment were at least as good as the growth and multiplication in a candle-jar. It is unclear why earlier investigators were unable to grow *P. falciparum* in sickle cells in low oxygen environment (Friedman, 1978; Pasvol et al., 1978). It could be because Medium 199, instead of RPMI 1640 growth medium, was used in one of the studies (Pasvol et al., 1978) or because the parasites were not allowed enough time to adapt to growth in sickle cells (Friedman, 1978; Pasvol et al., 1978). Although Friedman (1978) used RPMI 1640 medium, the cultures were terminated within the first 2–4 days. If the cultures had been maintained for a longer period, a few parasites would have, perhaps, survived and probably proceeded to grow normally. It is well known that, even in HbAA erythrocytes, it could take several weeks for some parasite strains to become well adapted to continuous growth in vitro. It has been our experience that even when most of the parasites in a new isolate appeared no longer viable, a few of them could still survive and eventually begin to multiply normally. The same thing could happen when there is a sudden change from one hemoglobin type to another. It is advisable to continue maintaining the culture as long as there is a parasite that appears somewhat healthy.

Most of the experiments reported here with HbAS were carried out with erythrocytes that were obtained from the same blood donor. However, we were also able to grow the three *P. falciparum* strains in erythrocytes from three other HbAS blood donors for at least 6 days in low oxygen environment. In one of the experiments in which *P. falciparum* HB3 strain was used, a 0.2% parasitemia on day 0 increased to 15% parasitemia on day 6, a 75-fold growth yield. After 6 days, the culture was used to infect HbAS erythrocytes from a different donor, and the parasites continued to grow normally in both low and high oxygen environments (data not shown).

In contrast with results obtained in HbAS erythrocytes, the parasite growth yield in HbSS erythrocytes was, in most cases, less in low oxygen environment than it was in the candle-jar (Fig. 5). It was also observed that HbSS erythrocytes sickled more readily and more extensively than sickle cell trait erythrocytes in the gas mixture that was used in the present experiments. Perhaps, the sickling damaged some of the parasites and interfered with their growth. In addition, it has been reported that HbSS erythrocytes contained excess amounts of potentially toxic FP molecules, which could also contribute to decreased parasite growth yield (Orjih et al., 1985).

The results of the experiments with erythrocytes from the sickle cell disease blood donors have been pooled in Fig. 5 because there was no clear difference in parasite growth among the different cell types. As has been stated in Section 2, the data were obtained with erythrocytes from the following sickle cell disease cases: two HbSS, one sickle cell beta-thalassemia and one HbSS with high HbF. Although beta-thalassemia and HbF have been reported to inhibit *P. falciparum* growth on their own, such inhibition has not been attributed to low oxygen tension (Pattanapanyasat et al., 1999; Pasvol et al., 1977). It is therefore likely that the difference between parasite growth in the sickle cell disease erythrocytes in low and high oxygen environments was mainly due to HbSS.

It has been suggested that parasite metabolism was inhibited in sickle cells in low oxygen environment (Friedman, 1978). We found here that, compared with the relatively high oxygen environment in the

candle-jar, the low oxygen tension in the gas mixture had no inhibitory effect on hemozoin production by *P. falciparum* parasites in HbAS erythrocytes. Nevertheless, hemozoin production by the parasites in HbSS erythrocytes was clearly less in low than in high oxygen environment (Fig. 7). Thus, low oxygen environment appears to interfere with hemoglobin catabolism and/or hemozoin synthesis in HbSS, but not HbAS erythrocytes.

The overall significance of the present work is that it has confirmed that *P. falciparum* can be grown in sickle cells at low oxygen tension in vitro, as has been reported earlier (Orjih, 1999). This is important because 2–5% oxygen is the standard partial oxygen tension commonly used for routine cultivation of *P. falciparum* in vitro, while the candle-jar method has virtually been abandoned in most of modern research laboratories. At present, virtually all the data on *P. falciparum* genetics have been from parasites in HbAA erythrocytes. However, some conditions in sickle cells, such as hemoglobin polymerization and excess ferriprotoporphyrin IX (Kuross et al., 1988), are quite different from those in HbAA erythrocytes, and probably could affect the genetics of malaria parasites. The work reported here may encourage some scientists to study the molecular biology of *P. falciparum* strains in sickle cells and compare it with parasites in HbAA erythrocytes.

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