Pyruvate kinase deficiency protects against malaria in humans

The association between inherited erythrocyte disorders in humans and resistance to malaria is well documented and includes enzyme deficiencies, hemoglobinopathies and membrane protein abnormalities. The association between pyruvate kinase (PK) deficiency and resistance to malaria has been demonstrated *in vivo* using a murine model[1] and it has been suggested that this phenomenon may extend to humans.

The human PK-LR gene codes for erythrocyte PK, which catalyzes the conversion of phosphoenolpyruvate to pyruvate. It is an essential step in anerobic glycolysis and is critical for ATP production in erythrocytes. There are more than 180 PK mutations, and clinical symptoms appear in patients who are homozygous or compound heterozygotes for an abnormal allele. The frequency of PK deficiency is highly variable, and prevalence studies in North America, Europe and parts of Asia found that the heterozygote allele frequencies ranged between 1% and 3.6%[2,3] in these regions. A study in African Americans suggested that the frequency of the heterozygote allele was 2.4 times more common in Africans than in Caucasians.[4]

Blood from a PK deficient patient and from the heterozygous parent was used for parasite culture experiments. The study was approved by the relevant ethics authority and both subjects gave their informed consent. Clinical and laboratory data are shown in Table 1. A G6PD screening test, hemoglobin electrophoresis and membrane protein studies were performed to exclude co-existing inherited erythrocyte disorders.

P. falciparum strain 3D7 was maintained in culture and synchronized according to standard methods.[5] Duplicate erythrocyte cultures of the homozygote (PK deficient patient), heterozygote (parent) and a control (healthy volunteer) were seeded on day one with parasite ring stages to give a hematocrit of 5% and a parasitemia of ~1.5%. Unseeded erythrocyte cultures for each subject were used as a *blank* to control for reticulocytes and autohemolysis. Parasitemia was monitored daily with Giemsa-stained blood smears and quantitated in duplicate by counting 5×10^4 cells by flow cytometry[6,7] for at least two complete cycles (six days). Briefly, a 2 µL aliquot of cells was added to freshly prepared thiazole orange (0.05 µg/mL phosphate buffered saline), incubated at 4°C for 30 mins. and analyzed on a FACSCalibur flow cytometer (BD Biosciences). Thiazole orange is a fluorescent dye that intercalates into nucleic acids and differentiates between non-nucleated erythrocytes and different parasite stages. A typical flow cytometry data set is shown in Figure 1A. Reticulocytes contain RNA and falsely elevate the parasitemia, which was corrected by subtracting blank readings from parasitized culture readings. The complete experiment was performed twice on different occasions.

Erythrocytes from the homozygous PK deficient patient demonstrated a dramatic resistance to *P. falciparum* infection (Figure 1B). Numerous *prickle cells*, typical of PK deficiency, were observed and these did not contain any parasites. Although the parasitemia in the heterozygote was slightly lower than the control, there was no statistically significant difference between them on day five (p=0.44) and six (p=0.55). Similar results were obtained in a second set of experiments.

Table 1. Clinical and laboratory data of a homozygous PK deficient patient, heterozygous parent and normal control.

Characteristic	*Homozygote*	*Heterozygote*	*Control*
Race	Caucasian	Caucasian	Caucasian
Hemoglobin (g/dL)	9.6	12.8	14.6
% reticulocytes	15.1%	5.0%	1.1%
PK activity (U/g Hb)	1.9	7.5	14.9
PK clinical phenotype	Moderate/severe	Asymptomatic	–
PK-LR genotype	1529A/A	1529G/A	1529G/G

The reference range for PK enzyme activity is 15±2 U/g Hb. The PK clinical phenotype was classified according to criteria set out by Zanella et al.[2] The G1529A point mutation results in an Arg510Gln amino acid change, which alters the conformation of the enzyme and impairs activity.

In vitro autohemolysis is a feature of inherited erythrocyte disorders and to asses its effect on cultures, the hemoglobin concentration in the supernatant of each culture was measured daily at 420nm. Over six days the hematocrit decreased from 5% to 4.9% (control), 4.8% (heterozygote) and 4.6% (homozygote), indicating that autohemolysis had minimal impact. Hemolysis due to parasitemia increased with increasing parasitemia (Figure 1C) but was, however, significantly less in the heterozygote compared with the control (p=0.0001 on day five; p=0.008 on day six). The reason for this is not clear, but indicates that a mild protective effect in the heterozygote cannot be excluded. The failure of the parasite-induced hemolysis to increase in the homozygote reflected the resistance to malaria in this subject.

The data presented in this paper demonstrate for the first time that PK deficient human erythrocytes are resistant to malaria infection *in vitro*, and support the *in vivo* findings in the murine model.[1] Two interesting questions arise from this study. Firstly, has malaria selected for the relatively high frequency of the abnormal PK allele? And secondly, how does PK deficiency exert its protective effect? The detrimental effect of PK deficiency on the host suggests that, like other inherited erythrocyte disorders, there was a historical driving force that maintained the polymorphic frequency of the abnormal allele. That malaria is the most likely driving force is proposed for several reasons: (i) this study has demonstrated that human PK deficient erythrocytes are resistant to malaria *in vitro*; (ii) *in vivo* protection has been shown in the mouse model;[1] (iii) until 1900 malaria was encountered in all regions where PK deficiency[8] has been reported; and (iv) malaria has been well-documented as a driving force behind other human erythrocyte disorders (Haldane's malaria hypothesis).

The mechanisms responsible for the resistance of PK deficient erythrocytes to malaria are unknown but may be due to ATP depletion, which impairs parasite invasion *in vitro*.[9] Decreased ATP leads to cross-linking of membrane proteins,[10] which may affect invasion, growth or exit of the

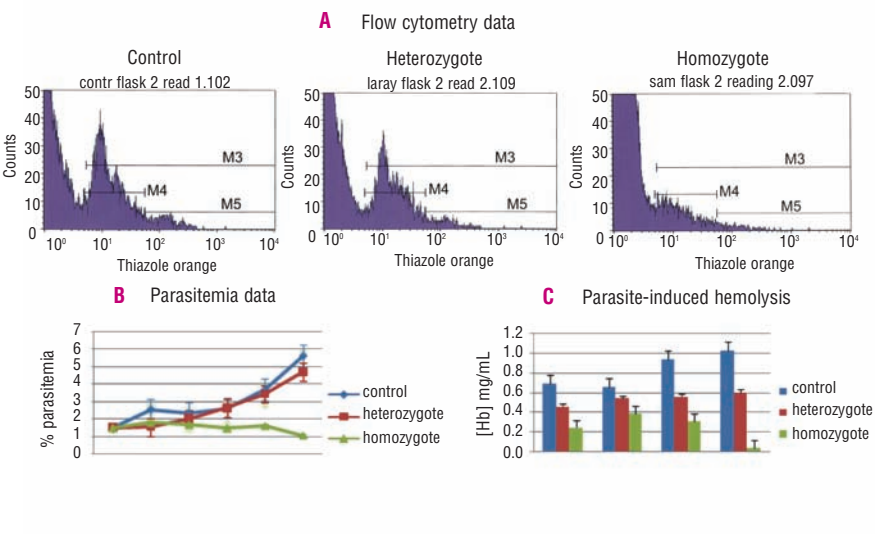

Figure 1. Parasitemia data. **A.** Flow cytometry data showing uncorrected total parasitemia readings for the control, heterozygote and PK deficient homozygote on day 5. M3–total parasitemia; M4–early trophozoites; M5–late trophozoites. **B.** Parasitemia (normalized to 1.5%) versus time. **C.** Parasite-induced hemolysis as measured by culture supernatant hemoglobin concentration ([Hb] mg/mL). Data in B and C are represented as a mean ± standard error of the mean (n=4).

parasite. Low ATP concentration also increases 2,3-diphosphoglycerate, which i) disrupts spectrin, actin and protein 4.1 interactions causing membrane instability[11] and impacting on intracellular parasite survival, and ii) inhibits G6PD leading to increased oxidative stress.[12] The scenario *in vivo* may include other factors such as splenic clearance, early phagocytosis and decreased cyto-adherence of parasitized erythrocytes. Our investigations have also demonstrated that the mutant PK activity decreased at elevated temperature (*data not shown*), suggesting that fever may exacerbate the low ATP concentration.

The *in vitro* data in humans presented here confirm the *in vivo* protection of PK deficiency demonstrated in the mouse model and lay the foundation for future clinical case control studies.

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