

BRIEF REPORT

Pyruvate Kinase Deficiency and Malaria

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SUMMARY

Malaria that is caused by *Plasmodium falciparum* is a significant global health problem. Genetic characteristics of the host influence the severity of disease and the ultimate outcome of infection, and there is evidence of coevolution of the plasmodium parasite with its host. In humans, pyruvate kinase deficiency is the second most common erythrocyte enzyme disorder. Here, we show that pyruvate kinase deficiency provides protection against infection and replication of *P. falciparum* in human erythrocytes, raising the possibility that mutant pyruvate kinase alleles may confer a protective advantage against malaria in human populations in areas where the disease is endemic.

MALARIA IS AN IMPORTANT PARASITIC DISEASE IN HUMANS, CAUSING an estimated 500 million clinical cases and more than 1 million deaths annually.¹ Disease control has been hampered by drug resistance in plasmodium parasites and by the lack of an effective vaccine.^{2,3} A better understanding of the pathogenesis of malaria, including the identification of innate or adaptive host defense mechanisms against the blood-stage parasite, may provide new targets for intervention in this disease. Such mechanisms may be manifested as genetic determinants of susceptibility in areas of endemic disease and during epidemics and as variations according to strain in mouse models of experimental infections.⁴⁻⁷

Genetic studies of susceptibility to malaria in a mouse model for the erythroid stage of the disease, with the use of infection with *P. chabaudi*, have localized a number of major loci affecting the extent of parasite replication at the peak of infection. Recombinant congenic mouse strains AcB55 and AcB61 are very resistant to infection with *P. chabaudi*; resistance in these strains segregates as a recessive monogenic trait caused by a mutation (Ile90Asn) in the gene for pyruvate kinase (*Pkfr*).^{8,9} The purpose of this study was to determine whether pyruvate kinase deficiency protects humans against malaria and to elucidate the molecular basis of a putative protective effect.

Pyruvate kinase catalyzes the rate-limiting step of glycolysis, converting phosphoenolpyruvate to pyruvate with the generation of one molecule of ATP. In the absence of mitochondria (which are lacking in mature erythrocytes), the enzyme is critical to energy production. Pyruvate kinase deficiency is the most frequent abnormality of the glycolytic pathway and, together with a deficiency in glucose-6-phosphate dehydrogenase (G6PD), is the most common cause of nonspherocytic hemolytic anemia. Pyruvate kinase deficiency is inherited as an autosomal recessive trait and is caused by loss-of-function mutations in *PKLR*. The prevalence of homozygous pyruvate kinase deficiency is estimated at 1 case per 20,000 persons; more than 158 mutations have been described.^{10,11}

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METHODS

SUBJECTS

From January 2006 to June 2007, subjects attending hematology clinics at the Toronto General Hospital and the Hospital for Sick Children who were identified as having pyruvate kinase deficiency on the basis of the clinical presentation and the results of an enzyme assay were eligible for enrollment in this study. Their asymptomatic relatives were also eligible for enrollment. The study was approved by the institutional review board at each center, and all subjects provided written informed consent.

We ruled out the presence of other hemolytic disorders by hemoglobin electrophoresis and assessment of the G6PD level. Subjects with homozygous pyruvate kinase deficiency included a 39-year-old man of Italian ancestry (Subject 1) and two women: 39-year-old Subject 2, also of Italian ancestry, and 19-year-old Subject 3, of French ancestry. All subjects had nonspherocytic anemia. Subject 3 was transfusion-dependent, and Subjects 1 and 2 had undergone splenectomy. A blood sample was drawn from Subject 3 before she underwent transfusion. The majority of humans with pyruvate kinase deficiency are compound heterozygotes with respect to the mutation of *PKLR*.^{10,12} The subjects in this study had not been previously genotyped to determine the genetic basis of their enzyme deficiency.

IDENTIFICATION OF *PKLR* MUTATION

Genomic DNA was isolated from the buffy coat of blood samples from subjects with pyruvate kinase deficiency (case subjects) and persons without pyruvate kinase deficiency (control subjects) with the use of proteinase K, phenol–chloroform extraction, and isopropanol precipitation. DNA (60 ng) — specifically, the 12 coding exons of *PKLR*, including intron–exon junctions — was used as a template for amplification by polymerase chain reaction (PCR), with 22 to 25 cycles at annealing temperatures ranging from 56° to 58°C. PCR products were purified and sequenced with the use of cycle sequencing with fluorescent nucleotides. Traces were analyzed with the use of BioEdit (www.mbio.ncsu.edu/BioEdit/bioedit.html), and all mutations were confirmed by sequence analysis.

PARASITE CULTURE

P. falciparum clones ITG and 3D7 (mycoplasma-free) were maintained in continuous culture.¹³

To assess parasite invasion and maturation, schizonts from synchronized cultures¹⁴ were mixed with erythrocytes from case subjects and control subjects, as described previously.¹⁵ In all samples, invasion of erythrocytes was assessed at 24 hours, 72 hours, and 120 hours, and maturation was assessed at 48 hours, 96 hours, and 144 hours.

PHAGOCYTOSIS ASSAY

Human monocytes were isolated and purified from the peripheral blood of healthy donors, as described previously.¹⁶ Thioglycollate-elicited macrophages were harvested from the peritoneal fluid of C57BL/6 mice.¹⁷ A total of 1.5×10^5 cells per well were plated on glass coverslips in 24-well plates and incubated for 5 days. All washed erythrocytes, including those infected with *P. falciparum* and those uninfected, underwent opsonization with 50% fresh autologous serum for 30 minutes at 37°C. Erythrocytes were then washed twice, resuspended at 10% hematocrit, and incubated with macrophages adhered to glass coverslips at a target-to-effector ratio of approximately 40:1. Phagocytosis assays were performed and assessed as described previously.¹⁸ All experiments were performed in duplicate and repeated at least three times.

ERYTHROCYTE MEMBRANE ANALYSIS

Bound hemichromes, IgG, and C3c fragments were measured as described previously.^{15,18} For ring-stage infected erythrocytes, the values were normalized to 100% parasitemia with the use of the following formula: $I = (\text{Tot} - N \times n) \div (1 - n)$, as described previously,¹⁵ in which I indicates the amount of bound IgG and C3c in 100% rings; Tot, the amount of bound IgG and C3c in the whole culture; N, the amount of bound IgG and C3c in erythrocytes without parasites; and n, the fraction of erythrocytes without parasites. For mature-stage infected erythrocytes, the percentage of parasitemia was 5 to 10%.

STATISTICAL ANALYSIS

We performed comparisons with the use of either Student's t-test (two-tailed) or the Mann–Whitney test.

RESULTS

***PKLR* MUTATIONS**

The characteristics of the three subjects with homozygous pyruvate kinase deficiency who pre-

sented with nonspherocytic hemolytic anemia are shown in Table 1. To confirm the diagnosis, we derived genomic DNA from the subjects with pyruvate kinase deficiency and from their asymptomatic relatives and sequenced all exons and intron-exon junctions of *PKLR*. We identified a homozygous G-to-A mutation at position 1269 at the 3' end of exon 9 in two related case subjects (Subjects 1 and 2), which has been previously described as a loss-of-function mutation. It is predicted to cause missplicing of *PKLR*, resulting in a shortened half-life of the messenger RNA transcript.^{10,11} Subject 3 was found to be homozygous for a single-base deletion at nucleotide position 823 in exon 7 of *PKLR*, leading to a frameshift and premature termination of the open-reading frame. The highly deleterious nature of this latter mutation may be responsible for the severe pyruvate kinase deficiency in Subject 3, who was transfusion-dependent. We also identified asymptomatic relatives of Subjects 1 and 2 who were heterozygous for the G-to-A mutation at position 1269 (Table 1). These relatives are designated as Subjects 4 and 5.

Erythrocytes from each of the subjects with a homozygous mutation (Subjects 1, 2, and 3) were infected in vitro (within 24 hours after collection) with two different *P. falciparum* isolates, 3D7 and ITG; 3D7 is sensitive to chloroquine, and ITG is resistant to chloroquine. We initially examined whether *P. falciparum* parasites invaded and matured as efficiently in erythrocytes from case subjects as in those from control (AA) subjects. The results of multiple invasion and maturation assays with erythrocytes from each subject with

a homozygous mutation in *PKLR* showed a reduction in the invasion of erythrocytes by *P. falciparum* parasites during three consecutive growth cycles, as compared with the invasion of erythrocytes from control subjects ($P=0.01$, $P<0.001$, and $P<0.001$ for the first, second, and third cycles, respectively) (Fig. 1A and 1B). Invasion assays that used erythrocytes from subjects carrying heterozygous mutations in *PKLR* (Subjects 4 and 5) did not reveal a significant defect in invasion (Fig. 1C). For both homozygotes and heterozygotes, no significant differences were observed in intracellular maturation (from the ring stage to the trophozoite stage) between erythrocytes from case subjects and those from control subjects (Fig. 1A and 1C). These results showed a reduced level of invasion of *P. falciparum* in erythrocytes from subjects with homozygous mutations. They also indicated that potential biochemical differences in the intracellular milieu, including the accumulation of glycolytic metabolic intermediates, did not cause a difference in parasite growth in erythrocytes between homozygotes and heterozygotes.

To further test the hypothesis that reduced invasion observed in erythrocytes from subjects with homozygous mutations is due to reduced fitness of the parasite, including altered development of merozoites, we examined erythrocyte invasion by merozoites derived from erythrocytes from case subjects. We observed that merozoites from such erythrocytes had normal levels of invasion and replication in erythrocytes from control subjects. (For details, see the Methods section and Table 1 of the Supplementary Appendix, available with the full text of this article at www.nejm.org.)

Table 1. Characteristics of Case Subjects with *PKLR* Mutations.*

Subject No.	Country of Origin	Age yr	Sex	Transfusion Dependent	Mutation	Effect	Hemoglobin† g/liter	Reticulocyte Count billion/liter	MCV fl	MCH pg
Homozygous mutation										
1	Italy	39	M	No	1269A	Splicing	90	885	117	39.1
2	Italy	40	F	No	1269A	Splicing	81	896	130	40.2
3	France	19	F	Yes	823delG	Frameshift	97	363	92.2	31.6
Heterozygous mutation										
4	Italy	44	F	No	1269A	Splicing	133	33	94.2	32.5
5	Italy	49	F	No	1269A	Splicing	130	108	92.5	31.3

* MCH denotes mean corpuscular hemoglobin, and MCV mean corpuscular volume.

† Reference ranges are 121 to 151 g per liter for women and 138 to 172 g per liter for men.

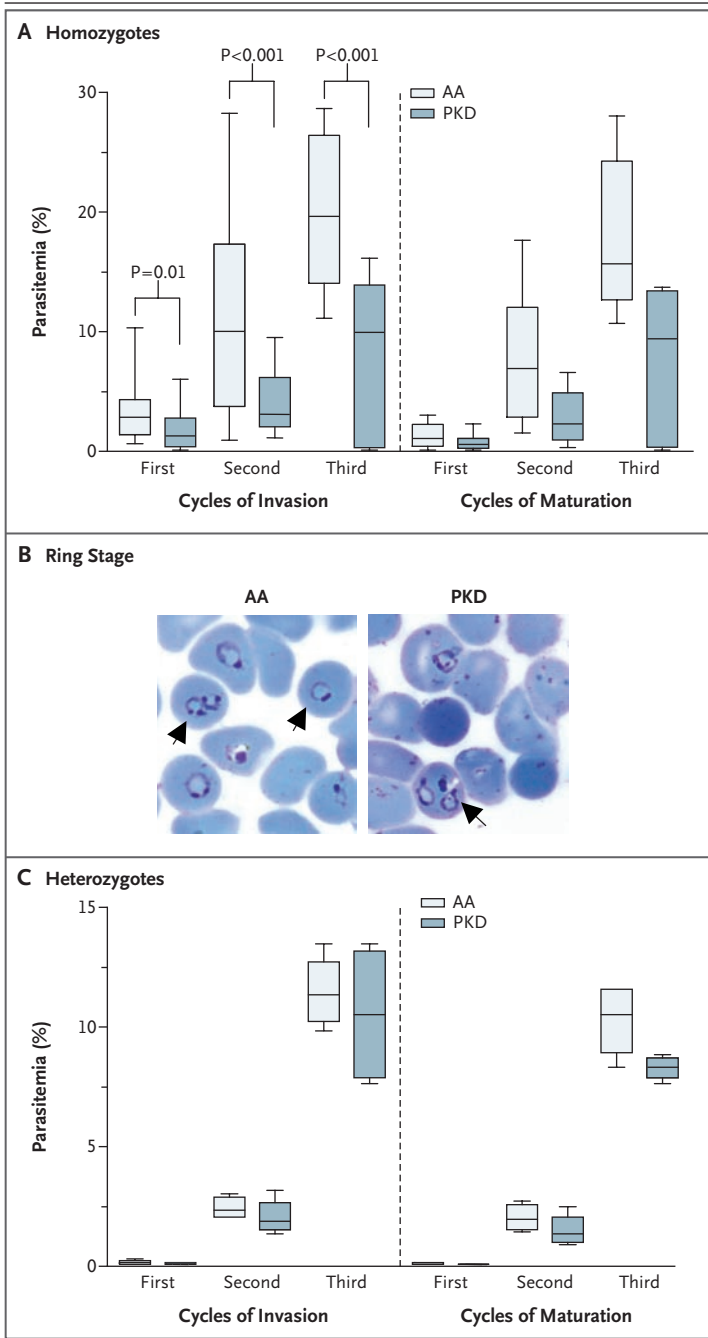


Figure 1. *Plasmodium falciparum* Invasion of Erythrocytes from Case Subjects and Control Subjects.

In Panel A, levels of invasion and maturation of *P. falciparum* are shown during three replication cycles in erythrocytes from control subjects (AA) and from case subjects with pyruvate kinase deficiency (PKD) who are homozygous for mutations in the pyruvate kinase gene. Data are presented as the combined results of at least two independent experiments performed with erythrocytes from each homozygous donor with PKD (Subjects 1, 2, and 3) and are shown as box-and-whiskers plots, representing interquartile and complete ranges, with the horizontal line in each box indicating the median level of parasitemia. P values are based on the Mann–Whitney test. Invasion is defined as the percentage of ring parasitemia, as measured 24 hours, 72 hours, and 120 hours after inoculation. Maturation is defined as the percentage of trophozoite parasitemia, as measured 48 hours, 96 hours, and 144 hours after inoculation. In Panel B, photomicrographs of blood smears of infected erythrocytes from control subjects and case subjects show ring forms of *P. falciparum* (arrows). In Panel C, there is no significant difference between control subjects and heterozygous case subjects in levels of invasion and maturation of *P. falciparum* in erythrocytes.

(ring-stage and mature-stage)–infected erythrocytes from case subjects and control subjects by macrophages derived from human and mouse monocytes (Fig. 2A and 2B). Phagocytosis of ring-stage–infected erythrocytes from case subjects with homozygous mutations (Subjects 1, 2, and 3) was markedly higher than phagocytosis of parasitemia-matched infected erythrocytes from control subjects (P<0.001). We also observed significantly enhanced clearance by macrophages of ring-stage–infected erythrocytes derived from asymptomatic relatives who were heterozygous for the *PKLR* mutation (P=0.003) (Fig. 2C).

To investigate the mechanistic basis of this difference, we measured the level of deposition of opsonins and hemichrome associated with the erythrocyte membrane.¹⁵ We correlated the enhanced phagocytic uptake of early-stage–infected erythrocytes from Subjects 1, 2, and 3 with increased levels of membrane-bound hemichromes (P<0.001), autologous IgG (P<0.001), and complement C3c fragments (P<0.001), as compared with ring-stage–infected erythrocytes from subjects with wild-type *PKLR* (Fig. 1 of the Supplementary Appendix). In contrast, macrophage uptake of mature-stage–infected erythrocytes from

Therefore, the observed reduction in parasite levels during in vitro cultivation in erythrocytes from case subjects appeared to be caused by an invasion defect attributable to a property of the erythrocytes.

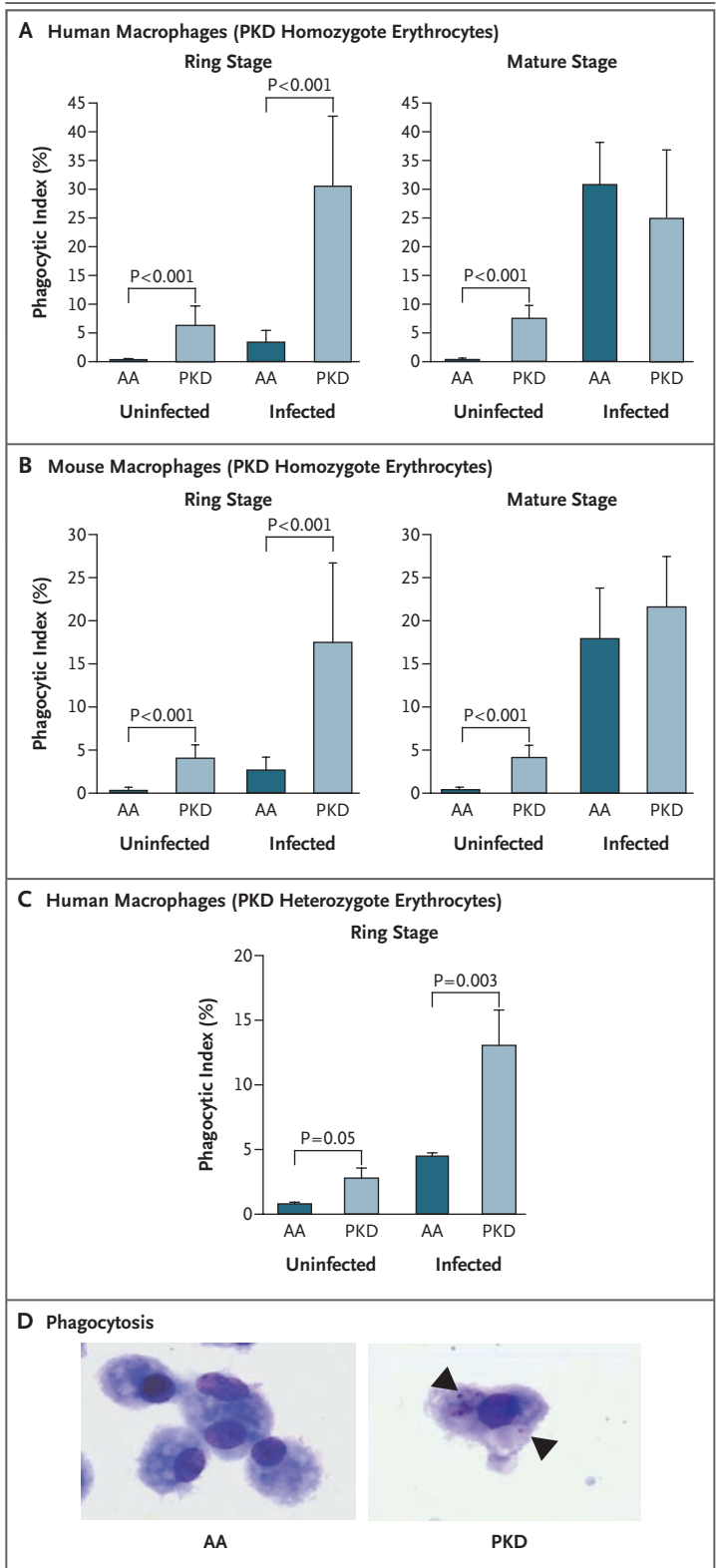
We examined phagocytic uptake of *P. falciparum*

Figure 2. Phagocytosis of Erythrocytes from Case Subjects and Control Subjects.

Levels of phagocytosis by human macrophages (Panel A) and mouse macrophages (Panel B) of erythrocytes from control subjects (AA) and homozygous case subjects with pyruvate kinase deficiency (PKD) that are either infected or uninfected with *Plasmodium falciparum* are shown in both the ring stage and the mature stage. Panel C shows levels of phagocytosis by human macrophages of erythrocytes from control subjects and asymptomatic heterozygous case subjects that are either infected or uninfected with *P. falciparum* at the ring stage or the mature stage. In all analyses, erythrocytes underwent opsonization with nonimmune human serum before phagocytosis. Data are presented as the pooled results of 10 independent experiments and are presented as means (\pm SD) (Panels A and B, subjects 1, 2, and 3; Panel C, subjects 4 and 5). All P values were calculated by Student's t-test. In Panel D, photomicrographs of phagocytosis show increased uptake in ring-stage-infected erythrocytes from subjects with PKD (arrowheads), as compared with control erythrocytes.

case subjects did not differ significantly from that of mature-stage-infected wild-type erythrocytes. At the mature stage of parasite development, erythrocytes from both case subjects and control subjects displayed marked membrane damage, as evidenced by similar levels of membrane deposition of complement C3c and IgG and by high and similar phagocytic uptake.

To determine whether macrophage uptake of ring-stage-infected erythrocytes from case subjects was mediated by IgG or complement, we carried out phagocytosis assays with complement-inactivated serum and in the presence of Fc-receptor blockade. We found that uptake was predominantly mediated by complement, since inactivation of complement (in autologous serum) caused a significant decrease in uptake, whereas Fc-receptor blockade had no significant effect (Fig. 1 of the Supplementary Appendix). As compared with erythrocytes from control subjects, uninfected erythrocytes from case subjects also had enhanced phagocytic uptake associated with increased deposition of hemichrome, IgG, and complement C3c, although at markedly lower levels than those in infected erythrocytes from case subjects. Together, these results showed that erythrocytes from case subjects that were infected with *P. falciparum* underwent more extensive phagocytosis than did infected erythrocytes from control sub-



jects, a process that occurs through a C3c-mediated pathway.

We examined single-nucleotide polymorphisms (SNPs) in *PKLR* in populations of varying ancestry (www.HapMap.org), including the Yoruba of Nigeria, where malaria is endemic. We did not observe differences in the prevalence of these SNPs in the various HapMap populations, although our analysis was inconclusive because of the relative paucity of informative *PKLR* SNPs in the HapMap database.

DISCUSSION

We have shown that pyruvate kinase deficiency has a protective effect against replication of the malarial parasite in human erythrocytes. We have described a dual mechanism for protection against *P. falciparum* in pyruvate kinase deficiency that included an invasion defect of erythrocytes from case subjects (observed in those with a homozygous mutation) and preferential macrophage clearance of ring-stage-infected erythrocytes from case subjects (observed in both homozygotes and heterozygotes). The pleiotropic effect of pyruvate kinase deficiency on parasite invasion (reduced) and phagocytosis of ring-stage-infected erythrocytes (enhanced) may provide protection against clinical malaria either by causing an overall reduction in the parasite burden or by reducing the number of erythrocytes infected with parasites in the trophozoite and schizont stages that are avail-

able to bind within microvascular beds of vital organs.¹⁹

In light of the poor overall health status and relative rarity of patients with pyruvate kinase deficiency who have homozygous mutations at *PKLR* (severe anemia with dependence on transfusion), it is unlikely that full-fledged pyruvate kinase deficiency is relevant to protection against malaria in the field. However, heterozygosity for partial or complete loss-of-function alleles or even compound heterozygosity for mild alleles with appropriate erythropoietic compensation may have little negative effect on overall fitness (including transmission of mutant alleles), while providing a modest but significant protective effect against malaria. Although speculative, this situation would be similar to that proposed for hemoglobinopathies (sickle cell and both α -thalassemia and β -thalassemia) and G6PD deficiency, in which similar mechanisms of protection that are associated with increased clearance of ring-stage-infected erythrocytes have been reported previously.^{6,15} Such a mechanism would be manifested as an increase in retention or prevalence of mutant *PKLR* alleles in regions where malaria is endemic, a hypothesis that can now be formally tested.

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No potential conflict of interest relevant to this article was reported.

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