Inhibitory Effect of a Fava Bean Component on the In Vitro Development of *Plasmodium falciparum* in Normal and Glucose-6-Phosphate Dehydrogenase Deficient Erythrocytes

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We examined the hypothesis that G-6-PD deficiency associated with fava bean ingestion confers resistance to malaria by studying the in vitro interactions between malaria parasites (*Plasmodium falciparum*), human erythrocytes with varying degrees of G-6-PD deficiency, and isouramil (IU), a fava bean extract that is known to cause oxidant stress and hemolysis of G-6-PD-deficient erythrocytes. Untreated G-6-PD-deficient and normal erythrocytes supported the in vitro growth of *P. falciparum* equally well. However, after pretreatment with IU, G-6-PD-deficient erythrocytes did not support parasite growth in vitro, whereas growth remained high in normal erythrocytes. Parasite growth was proportional to the G-6-PD activity of the IU-treated erythrocytes. In contrast, when parasitized erythrocytes were exposed to IU, parasites even in normal erythrocytes were destroyed. Ring forms were much less sensitive than late trophozoites and schizonts. The results suggest that there are two modes by which IU affects the development of *P. falciparum* and demonstrate in vitro that G-6-PD deficiency confers resistance against malaria under conditions of fava-bean-associated oxidant stress.

**Materials and Methods**

**Parasite.** *Plasmodium falciparum* was cultured according to the method of Trager and Jensen. The parasites were grown in 75 sq cm disposable plastic flasks in an atmosphere of 90% N₂, 5% O₂, and 5% CO₂.

**RBC suspensions enriched in parasites.** Suspensions enriched in trophozoites and schizonts were obtained by the gelatin sedimentation technique of Jensen from cultures containing 10%–15% parasitized erythrocytes. The upper layer, consisting of 60%–85% trophozoites and schizonts, was used in experiments requiring a minimal amount of noninfected erythrocytes in the inoculum.

**Synchronization of cultures.** Parasitized red blood cells were separated with gelatin as described above. The upper layer containing trophozoites and schizonts was washed, mixed with 6 volumes of nonparasitized erythrocytes to yield a 5% final cell suspension and returned to culture. Seventeen hours later the erythrocytes were washed and resuspended in 5% sorbitol in distilled water for 15 min according to Lambros and Vanderberg. The resulting suspension containing only ring forms was incubated for an additional 14 hr, after which the sorbitol treatment was repeated. Fresh erythrocytes were washed and added to adjust the parasitemia to 2%. The parasites still grew synchronously 5 days later.

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**Blood samples.** Blood was collected in ACD from normal donors and G-6-PD-deficient donors and stored at 4°C. The experiments were initiated within 48 hr after collection.

**G-6-PD activity.** The activity of G-6-PD was measured according to Bergmeyer.15

**Preparation of IU.** IU was synthesized according to Bien et al.,16 dissolved (2 mM) in deoxygenated PBS pH 7 solution, kept sealed under N₂ on ice, and used within 30 min after preparation.

**IU treatment.** Erythrocytes were washed twice in PBS and resuspended in IU solution to give a final concentration of 1 mM. A control for every sample was carried out using identical treatment with PBS. Blood suspensions were incubated at 37°C with gentle shaking for 30 min. The red blood cells were then washed with RPMI 1640 medium and suspended in the medium used for parasite cultures to give a concentration of 2.5% v/v red blood cells. A control erythrocytes with normal activity. Figure IA contained by nontreated normal erythrocytes added with the infected normal erythrocytes and then were treated with IU as described above.

**RESULTS**

The G-6-PD activity in the blood of two males, M.Z. and A.B., was less than 2% of a normal control, indicating that they were hemizygous for G-6-PD deficiency. Members of the family of A.B. were examined and their G-6-PD values were as follows: father, normal; mother, 50%; daughter, 40%; son A.B., <2%; son Y.B., normal. It was assumed, therefore, that the females were heterozygotes for G-6-PD.

The development of *P. falciparum* in G-6-PD-deficient erythrocytes and in the normal controls was measured after pretreatment of the erythrocytes with IU. Figure 1 shows the development of the parasite in the G-6-PD-deficient erythrocytes of M.Z. and in control erythrocytes with normal activity. Figure 1A shows that the development of *P. falciparum* in the normal erythrocytes was not affected by pretreatment of the red blood cells by IU. In both cases the parasitemia reached about 10% on the third day. Analogously, the development of the parasite in G-6-PD deficient erythrocytes (Fig. 1B) exhibited a similar pattern. In contrast, pretreatment of the G-6-PD-deficient cells with IU rendered them nonsuitable for parasite growth.

Figure 2 displays the daily levels of parasitemia in the erythrocytes of A.B. and his family. Red blood cells, that were not treated with IU from each donor of this family supported the growth of *P. falciparum* to considerable levels, above 14% parasitemia. This pattern was altered when the erythrocytes had been pretreated with IU. While treatment of erythrocytes from the father and son Y.B. (normal G-6-PD activity) only slightly reduced the parasitemia, treatment of A.B. (<2% activity) almost completely inhibited the growth of the parasites. The two females (40%–50% G-6-PD activity) supported the growth of the plasmodia to intermediate levels. Thus, in IU-treated erythrocytes the development of *P. falciparum* was proportional to the enzymatic activity of G-6-PD.

Unlike the previous experiments in which the erythrocytes were pretreated with IU and then infected with plasmodia, a new set of experiments was conducted in which already parasitized erythrocytes were treated. The effect of IU on parasitized erythrocytes was evaluated with nonsynchronized cultures and with synchronized cultures containing about 99% ring forms or 99% trophozoites (Fig. 3). Nonsynchronized cultures containing mixed stages (Fig. 3, top) showed retarded development for the first 3 days, after which the treated and control groups reached the same level of parasitemia. IU treatment had less pronounced effect on the ring forms (Fig. 3, bottom). The trophozoites were most sensitive: following the IU treatment, parasitemias rapidly declined to zero (Fig. 3, middle).

**DISCUSSION**

Epidemiologic studies have indicated that G-6-PD deficiency confers some resistance to malaria.1,2 This finding is in line with the general biologic theory that
genetic disorders of red blood cells, like hemoglobinopathies, are expressed as balanced polymorphism. Such balanced polymorphism would need to stem from a selective force in biologic evolution, which could provide an advantage to the heterozygote phenotype over the normal and deficient homozygote.

The suggestion that G-6-PD deficiency confers resistance to malaria has been challenged by other epidemiologic studies. It has been postulated that the protection against malaria occurs only in areas where favism exists.

We have shown in vitro that both G-6-PD-deficient and normal erythrocytes supported the growth of *P. falciparum* to similar levels. In contrast, G-6-PD-deficient erythrocytes, after treatment with the favism-inducing agent IU, were unable to support the parasites' growth. The same treatment of normal erythrocytes did not affect the growth. Erythrocytes from heterozygote females, where the G-6-PD level was about half that of normal, supported the growth of the parasite to intermediate levels. Thus, in IU-treated erythrocytes, parasitemias were proportional to the G-6-PD activity.

Isouramyl has been shown to produce deleterious effects on G-6-PD-deficient erythrocytes as compared with normal erythrocytes. These effects include irreversible depletion of cellular GSH, elevated levels of methemoglobin, a shortened lifespan of the treated erythrocytes in vitro, and increased susceptibility of the erythrocytes to phagocytosis in vitro. IU is a reductone characterized by high reducing capacity, which leads to the formation of superoxide radicals, hydrogen peroxide, and hydroxyl radicals. Thus, it exerts a massive oxidative stress on the erythrocyte. The irreversible alterations that have taken place in the deficient erythrocytes affect the parasite and are reflected by reduced parasitemias.

The effect on heterozygote erythrocytes can be explained by the fact that heterozygote subjects have two populations of erythrocytes, one with normal G-6-PD activity and the other enzyme deficient. The infective stage, the merozoite, once emerged in the medium, has equal chances to meet either a normal or
G-6-PD-deficient cell. The parasite may be unable to discriminate externally between the different cells, it adheres to or even penetrates equally into both cells but the deficient cells cannot support the growth. Alternatively, it is possible that the parasite adheres equally to both types of cells but is able to discriminate between the suitable and the nonsuitable host cell and detach from the nonsuitable one. During this process the parasite may become depleted of all its energy and exhaust its lifespan outside the red blood cells while searching for the appropriate host. This would effectively reduce the parasitemia.

The mechanism of interference with parasite development in treated erythrocytes has to be further investigated, and at this stage, it is not clear whether the effect is on attachment or penetration or intracellular development of the parasite.

While pretreatment with IU did not change the ability of normal erythrocytes to support the growth of plasmodia, it had a destructive effect on parasites when infected blood was exposed to IU. Young stages (ring forms) were much less sensitive than trophozoites and schizonts. The intracellular parasites also exert oxidant stress on the infected erythrocyte. It may be possible that the combined stress on the cell caused by the IU treatment and by the mature intracellular parasites is enough to inhibit the development of parasites even in normal erythrocytes. The G-6-PD-deficient parasitized erythrocyte is probably even more susceptible to such combined damage. Similar process occurring in vivo after ingestion of fava beans may lead to early sequestration of a parasitized erythrocyte before the parasite completes its cycle.

It would appear from the in vitro results presented here that there are at least two separate mechanisms by which the favism-inducing agent, isouramil, affects the development of the malarial parasite *P. falciparum*. These in vitro effects suggest that normal individuals may be partially protected and G-6-PD-deficient individuals more completely protected against malaria by the ingestion of IU in fava beans.

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