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SUSCEPTIBILITY OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE DEFICIENT RED CELLS TO PRIMAQUINE ENANTIOMERS AND TWO PUTATIVE METABOLITES—I

EFFECT ON REDUCED GLUTATHIONE, METHEMOGLOBIN CONTENT AND RELEASE OF HEMOGLOBIN

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Abstract—The effects of the primaquine (PQ) enantiomers, (+)PQ and (−)PQ, and two putative metabolites [5-hydroxyprimaquine (5HPQ) and 6-desmethyl-5-hydroxyprimaquine (6D5HPQ)] on methemoglobin (Met Hb) and glutathione content and release of hemoglobin into plasma from glucose-6-phosphate dehydrogenase (G-6-PD) deficient red cells were studied *in vitro*. The results show that a 1.5 mM concentration of (−)PQ produced a significantly greater increase in Met Hb content and decrease in reduced glutathione (GSH) level than did (+)PQ. However, the release of plasma hemoglobin was greater with (+)PQ than with (−)PQ. The hydroxy derivatives of primaquine, 5HPQ and 6D5HPQ, were significantly more active than PQ. Their individual effects differed; whereas 5HPQ produced significantly greater reduction in GSH compared to 6D5HPQ, the effect of 6D5HPQ on Met Hb content and release of plasma hemoglobin was greater than that of 5HPQ. The qualitative effects of these compounds on normal, heterozygous and hemizygous G-6-PD deficient red cells were similar, but quantitatively the effects were greatest on hemizygous G-6-PD deficient cells and intermediate on heterozygous cells.

Glucose-6-phosphate dehydrogenase (G-6-PD)§ deficiency is the most common enzyme deficiency in humans. It is estimated that around 100 million people suffer from this genetic abnormality [1]. Individuals with G-6-PD deficiency develop hemolytic anemia after exposure to a variety of drugs, notably primaquine [2]. Several different types of G-6-PD variants have been described [3], and the susceptibility of each variant to individual drugs is variable [4, 5].

Primaquine (PQ) is an 8-aminoquinoline. It is the drug-of-choice for radical treatment of vivax and ovale malaria [6]. These parasites infect hundreds of millions of people each year [7]. The usefulness of primaquine is limited by its ability to produce hemolysis in G-6-PD deficient individuals when given in therapeutic dosage [8]. Most investigators have suggested that oxidation of hemoglobin and other red cell proteins is the principal toxic effect of PQ [9–13], but this has been questioned recently [14, 15]. Our results support the hypothesis that the effect of PQ and its metabolites on red cell membranes may be independent of hemoglobin oxidation. Further, we report that two enantiomers of primaquine, (+)PQ and (−)PQ, have differential toxicities, and the putative hydroxy-metabolites of primaquine are several-fold more toxic than primaquine itself.

MATERIALS AND METHODS

Fresh human blood (20 ml) from normal healthy volunteers and patients with G-6-PD deficiency (hemizygous males) was drawn in EDTA-treated vials. Heterozygotes were selected from amongst mothers and daughters of the G-6-PD deficient subjects on the basis of quantitative assay of G-6-PD. G-6-PD activity was quantitated in 2-ml aliquots by the method of Zinkham *et al.* [16].

A 1.0-ml aliquot of whole blood was incubated with primaquine, PQ enantiomers (+ or −), or one of the two putative metabolites (5HPQ and 6D5HPQ) at a final concentration of 1.5 mM, in triplicate samples. This concentration is similar to that used in several other studies [17–19]. Equal amounts of saline, substituted for drug, served as controls. Incubation was for 1 hr at 37° in a water bath. After incubation, two 0.4-ml aliquots were taken from each tube for separate estimation of reduced glutathione (GSH) and methemoglobin (Met Hb) content. The remaining blood was centri-

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§ Abbreviations: G-6-PD, glucose-6-phosphate dehydrogenase; PQ, primaquine; (+)PQ, primaquine dextrorotatory; (−)PQ, primaquine levorotatory; 5HPQ, 5-hydroxyprimaquine; 6D5HPQ, 6-desmethyl-5-hydroxyprimaquine; Met Hb, methemoglobin; and GSH, reduced glutathione.

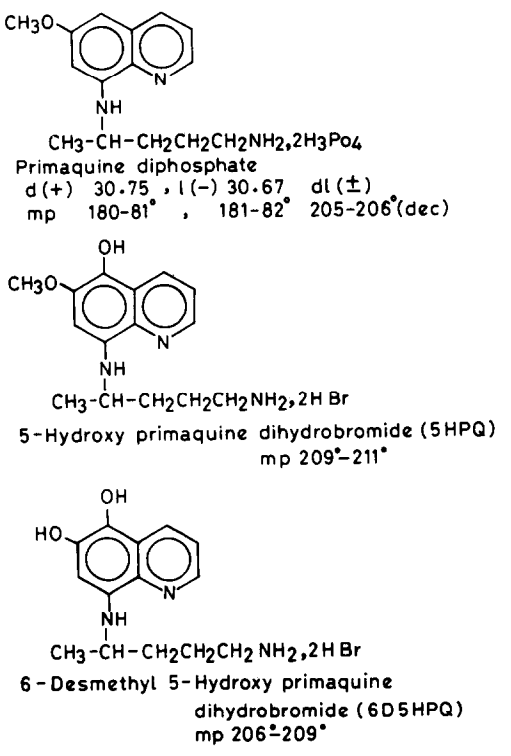

Fig. 1. Structural formulae, optical rotational data, and melting points of the compounds used in the study.

fuged at 2000 rpm for 15 min to obtain supernatant fluid for measurement of released plasma hemoglobin. All assays were done according to methods described by Dacie and Lewis [20]. The studies were carried out on three to six groups of normal, heterozygous, and hemizygous G-6-PD deficient individuals.

Source of primaquine enantiomers and its metabolites. (+)Primaquine and (−)primaquine enantiomers, and metabolites, 5-hydroxyprimaquine (5HPQ) and 6-desmethyl-5-hydroxyprimaquine (6D5HPQ), were synthesised in this laboratory (R. C. G.). The structural formulae, optical rotational data for enantiomers, and melting points of the compounds used in the study are shown in Fig. 1. The values for enantiomers compare well with those reported by Caroll *et al.* [21]. The metabolites were synthesised by selective demethylation of 5-methoxyprimaquine using 48% hydrobromic acid. The products showed single peaks on HPLC and have been characterised by spectroscopic analysis.

RESULTS

G-6-PD activity. The G-6-PD activity in crude hemolysates of normal red cells was 0.47 ± 0.07 units/mg of protein. Corresponding values in heterozygous and hemizygous affected individuals were 0.127 ± 0.04 and 0.041 ± 0.011 units respectively.

Reduced glutathione. Table 1 shows that basal levels of reduced glutathione in G-6-PD deficient red cells were significantly lower compared to normal erythrocytes ($P < 0.001$). Values in heterozygotes were intermediate. These results indicate that baseline levels of GSH in G-6-PD deficient individuals are lower.

Data in Table 2 show that PQ lowered GSH content by 7, 18 and 30% compared to saline controls in normal, heterozygous and hemizygous G-6-PD deficient red cells respectively. Comparable decreases by (−)PQ were 22, 28 and 47% and by (+)PQ were 16, 12 and 27%. Statistically the effect of (−)PQ was greater than that of (+)PQ on G-6-PD deficient red cells ($P < 0.001$) as well as on heterozygous ($P < 0.001$) and normal ($P < 0.05$) red cells.

The putative metabolites of PQ, 5HPQ and 6D5HPQ, lowered GSH to a considerably greater extent than did primaquine itself ($P < 0.001$). The effect was most marked on G-6-PD deficient red cells; GSH levels were lowered by 35–45% in normal red cells, 41–46% in heterozygous red cells and 64–80% in hemizygous G-6-PD deficient red cells. Of the two compounds tested, 5HPQ caused an apparent greater decrease of GSH than did 6D5HPQ; statistically, however, the difference was significant only in G-6-PD deficient red cells ($P < 0.05$).

Methemoglobin. The basal Met Hb levels in normal, heterozygous and hemizygous G-6-PD deficient red blood cells were not significantly different ($P > 0.05$) (Table 1).

On incubation with PQ, (+)PQ and (−)PQ, Met Hb levels increased respectively, to 13.8, 9.5 and 10.7% in normal red cells, 16.9, 11.9 and 15.8% in heterozygotes and 17.3, 10.6 and 14.2% in hemizygous deficient red cells (Table 3). PQ produced more Met Hb than its enantiomers in all the subjects tested ($P < 0.05$), whereas (−)PQ produced sig-

Table 1. Basal levels of GSH, Met Hb and plasma Hb

	GSH (mg/L red cells)	Met Hb (% hemoglobin)	Plasma Hb (mg/L red cells)
Normal (N)	665.7 ± 91.8	4.0 ± 1.5	8.3 ± 4.3
Heterozygous (D)	563.8 ± 109.7 ($P < 0.001$)*	5.6 ± 1.7 ($P > 0.05$)	9.8 ± 4.9 ($P > 0.05$)
Hemizygous (D)	446.2 ± 91.0 ($P < 0.001$)	5.6 ± 1.1 ($P > 0.05$)	13.6 ± 8.5 ($P > 0.05$)

Each value is the mean ± SD, N = 6.
* P value of N vs D.

Table 2. Effects of primaquine (PQ), its enantiomers, and two putative metabolites on reduced glutathione levels

Compound	Glutathione level (% of saline)		
	Normal	Heterozygous	Deficient
Saline*	100	100	100
PQ	92.7 ± 4.1	82.4 ± 3.2	70.3 ± 6.8
(+)PQ	83.6 ± 11.9	88.2 ± 6.6	72.5 ± 10.5
(−)PQ	78.2 ± 9.1	72.2 ± 9.9	52.5 ± 14.3
	(P < 0.05)†	(P < 0.001)	(P < 0.001)
5HPQ	55.3 ± 12.1	54.8 ± 3.8	20.3 ± 7.5
6D5HPQ	65.0 ± 9.8	59.7 ± 1.5	36.3 ± 9.1
	(P > 0.05)‡	(P > 0.05)	(P < 0.05)
	(P < 0.001)§	(P < 0.001)	(P < 0.001)

Each value is the mean ± SD of triplicate assays in three to six subjects. The concentration of all compounds used was 1.5 mM. Incubation was for 1 hr at 37° in a water bath.

* Absolute values of GSH were: normal, 665.7 ± 91.8, heterozygous, 563.8 ± 109.7, and deficient, 446.2 ± 91.0 mg/L red cells.

† P value of (+)PQ vs (−)PQ.

‡ P value of 5HPQ vs 6D5HPQ.

§ P value of PQ vs metabolites.

Table 3. Effects of primaquine (PQ), its enantiomers, and two putative metabolites on methemoglobin levels

Compound	Methemoglobin level (%)		
	Normal	Heterozygous	Deficient
Saline	4.0 ± 1.5	5.6 ± 1.7	5.6 ± 1.1
PQ	13.8 ± 2.5	16.9 ± 5.1	17.3 ± 4.3
(+)PQ	9.5 ± 4.7	11.9 ± 4.5	10.6 ± 3.8
(−)PQ	10.7 ± 4.9	15.8 ± 5.1	14.2 ± 5.7
	(P > 0.05)*	(P < 0.05)	(P < 0.05)
5HPQ	33.5 ± 1.5	33.4 ± 4.0	51.9 ± 1.9
6D5HPQ	40.8 ± 4.3	51.2 ± 2.7	79.5 ± 4.6
	(P < 0.05)†	(P < 0.01)	(P < 0.01)
	(P < 0.001)‡	(P < 0.001)	(P < 0.001)

Each value is the mean ± SD of three to six subjects. See Table 1 for basal values and the legend of Table 2 for experimental conditions.

* P value of (+)PQ vs (−)PQ.

† P value of 5HPQ vs 6D5HPQ.

‡ P value of PQ vs metabolites.

nificantly greater amounts of Met Hb than (+)PQ in heterozygous and hemizygous G-6-PD deficient red cells ($P < 0.05$). The difference between the effects of (−)PQ and (+)PQ on normal red cells was not significant ($P > 0.05$).

Compared to primaquine, the putative metabolites produced a five to seven times greater increase in Met Hb in red cells from all the individuals, the effect being significantly higher in hemizygous deficient red cells ($P < 0.001$). However, unlike the effect on GSH, 6D5HPQ produced a greater amount of Met Hb in normal red cells than 5HPQ ($P < 0.05$) as well as in heterozygous and hemizygous G-6-PD deficient red cells ($P < 0.01$).

Hemoglobin release. Normal, heterozygous and hemizygous G-6-PD deficient red cells, without any drug, released small amounts of hemoglobin in the supernatant fraction during 1 hr of incubation at 37° (Table 1). The release was significantly greater when red cells were incubated with PQ, its enantiomers and its putative metabolites (Table 4). The effect of (+)PQ was greater than that of (−)PQ. The differences were statistically significant among all three groups but were most marked on red cells from hemizygous G-6-PD deficient individuals.

DISCUSSION

This study showed that (−)PQ produced greater oxidation in red cells than (+)PQ. This is reflected in significantly greater production of Met Hb ($P < 0.05$) and reduction in GSH content by (−)PQ than by (+)PQ ($P < 0.01$). On the other hand, release of plasma hemoglobin was greater with (+)PQ than with (−)PQ ($P < 0.01$). Further, the effects of 5HPQ

Table 4. Effects of primaquine (PQ), its enantiomers, and two putative metabolites on plasma hemoglobin release

Compound	Plasma hemoglobin level (mg/L)		
	Normal	Heterozygous	Deficient
Saline	8.3 ± 4.3	9.8 ± 4.9	13.6 ± 8.5
PQ	10.3 ± 2.3	13.9 ± 1.1	17.1 ± 1.3
(+)PQ	21.5 ± 7.2	28.6 ± 8.5	36.1 ± 14.3
(−)PQ	16.7 ± 2.6	23.2 ± 6.2	25.0 ± 14.9
	($P < 0.05$)*	($P < 0.05$)	($P < 0.01$)
5HPQ	42.1 ± 4.3	51.9 ± 3.5	85.2 ± 4.3
6D5HPQ	47.5 ± 3.5	80.3 ± 3.5	114.3 ± 7.2
	($P > 0.05$)†	($P < 0.01$)	($P < 0.01$)
	($P < 0.001$)‡	($P < 0.001$)	($P < 0.001$)

Each value is the mean ± SD of three to six subjects. See Table 1 for control values and the legend of Table 2 for experimental conditions.

* P value of (+)PQ vs (−)PQ.

† P value of 5HPQ vs 6D5HPQ.

‡ P value of PQ vs metabolites.

and 6D5HPQ on GSH content and plasma hemoglobin release were discordant, i.e. while 5HPQ produced a greater decrease in GSH ($P < 0.05$), 6D5HPQ produced a greater release of plasma hemoglobin ($P < 0.01$). The effects of both the PQ enantiomers and putative metabolites of PQ on plasma hemoglobin release were significantly higher in hemizygous G-6-PD deficient red cells than in normal red cells and the values in heterozygotes were in the intermediate range, indicating that release of plasma hemoglobin correlated with severity of G-6-PD deficiency.

The discordance between the effects on GSH content and hemoglobin release indicates that leakiness of G-6-PD deficient red cells may be independent of oxidative damage to red cells. Baird *et al.* [14, 15], with an independent approach, reached identical conclusions. They showed that hydroxy derivatives of primaquine stimulate the hexose monophosphate shunt of red cells without causing proteolysis, the latter being taken as an indicator of oxidation of red cell protein [22]. Baird *et al.* [14, 15] have gone to the extent of completely negating the role of oxidative damage to red cell proteins. However, our study indicates that with definite lowering of GSH content and increase in Met Hb percentage by PQ enantiomers and its putative metabolites proportional to the degree of G-6-PD deficiency, the role of oxidative damage in overall survival of G-6-PD deficient red cells cannot be excluded. The validity of proteolytic activity as a measure of oxidative damage to red cell proteins compared to formation of Met Hb and reduction of GSH needs to be evaluated further before the conclusions of Baird *et al.* [14, 15] can be fully accepted.

The effects of 5HPQ and 6D5HPQ on GSH and Met Hb content were also found to be discordant in this study. Whereas 5HPQ produced a greater decrease in GSH, 6D5HPQ produced a greater increase in Met Hb in all three groups of individuals studied. It is speculated that 5HPQ may have a greater affinity to cellular proteins other than Hb, oxidation of which also leads to a decrease in GSH content. This might explain the apparent lack of a relationship between Met Hb and GSH levels observed in this study.

Differences in the toxicities of (+)PQ and (−)PQ have been reported by Schmidt *et al.* [23]. However, the toxicities of the two enantiomers were different between Rhesus monkeys and mice. Whereas in Rhesus monkey (−)PQ was more toxic than (+)PQ, the opposite was observed in mice. In the present study we have found that the effects of the two enantiomers, (−)PQ and (+)PQ, on oxidative indices and membrane leakiness of human red cells were discordant. Since at present the importance of the two parameters, viz. oxidative indices and membrane leakiness, with respect to *in vivo* hemolysis is not yet known, it is difficult to estimate which of the two enantiomers may be more toxic in humans. However, the findings suggest that the enantiomers may have differential toxicities in humans as well.

Compared to PQ, the putative hydroxy metabolites of primaquine (5HPQ and 6D5HPQ) produced significantly greater oxidation and red cell membrane leakiness in G-6-PD deficient red cells. This was also reported by Allahyari *et al.* [24] and Baird *et al.* [14, 15]. These metabolites are believed to occur *in vivo* [25, 26]. A suitable modification of the structure of the parent molecule which obviates production of reactive metabolites *in vivo* may be useful if such metabolites are not related to clinical efficacy. Increased intracellular oxidation by the metabolites tested in this study shows that hydroxyl groups at the 5 and 6 positions greatly enhance this activity. These and similar putative metabolites, therefore, represent likely candidates for studies seeking non-toxic primaquine substitutes giving therapeutic activity against the exoerythrocytic stages of *Plasmodium vivax* and *P. ovale*.

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