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

II. EFFECT ON RED BLOOD CELL MEMBRANE, LIPID PEROXIDATION, MC-540 STAINING, AND SCANNING ELECTRON MICROSCOPIC STUDIES

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Abstract—The effects of primaquine (PQ), its enantiomers [(+)PQ,(−)PQ] and hydroxy metabolites [5-hydroxyprimaquine (5HPQ) and 6-desmethyl-5-hydroxyprimaquine (6D5HPQ)] on cell membranes of glucose-6-phosphate dehydrogenase (G-6-PD) deficient red cells were studied *in vitro*. There was no significant effect of PQ on the malonyldialdehyde (MDA) content of normal and heterozygous red cells, but it caused a significant increase in MDA in G-6-PD deficient red cells ($P < 0.05$). There was no noticeable difference between the effects of the two enantiomers on this variable ($P > 0.05$). Compared to PQ, the hydroxy metabolites produced a significantly greater increase in MDA in all the groups studied ($P < 0.001$). Of the two hydroxy metabolites, 6D5HPQ was more toxic than 5HPQ. Staining with MC 540 showed that exposure to PQ, its enantiomers and two putative metabolites produced significant fluorescence, indicating that the drug produces marked alterations in membrane fluidity. Although the fluorescence was seen both in normal and heterozygous cells, the effect was marked in hemizygous deficient red cells ($P < 0.001$). Scanning electron microscopic (SEM) studies revealed that PQ enantiomers had a stomatocytic effect on red cells of normal, heterozygous and hemizygous G-6-PD deficient red cells, whereas the putative metabolites had an echinocytic effect. The effects were most pronounced in G-6-PD deficient red cells.

It has been shown that glucose-6-phosphate dehydrogenase (G-6-PD) deficient erythrocytes are sensitive to priamaquine (PQ) [1] and a variety of other compounds [2]. However, the mechanism of hemolysis is not established [3–7]. Several investigators consider that oxidative damage to hemoglobin plays an important role in determining red cell survival. The formation of covalent bonds between heme and drugs [5], the generation of free radicals and singlet oxygen along with H_2O_2 [6], an increase in the amount of methemoglobin (MetHb), a decrease in the amount of reduced glutathione (GSH) and binding of precipitated hemoglobin (Heinz bodies) to cell membrane have all been proposed. On the other hand, direct action of oxidant drugs on the cell membrane has been claimed to be equally important [8, 9]. We have reported previously that there is a discordance between the rise in MetHb, the decrease in GSH and plasma leakiness as determined by the quantitation of plasma hemoglobin when G-6-PD deficient red cells are treated with PQ, its enantiomers and hydroxy metabolites [10].

This report describes our observations on lipid peroxidation, membrane fluidity, and cell membrane morphology in G-6-PD deficient red cells using these compounds.

MATERIALS AND METHODS

Selection of subjects. Fresh human blood (20 mL) from healthy volunteers and patients with G-6-PD deficiency (hemizygous) was drawn in EDTA-treated vials. Heterozygotes were selected amongst mothers and daughters of G-6-PD deficient subjects based on a G-6-PD quantitative assay. In an aliquot of 2 mL, a quantitative assay of G-6-PD was carried out as described by Zinkham *et al.* [11]. Four subjects from each category were studied. For electron microscopic studies, the percentage of cells was recorded after counting ten fields.

Source of primaquine enantiomers and its metabolites. (+)Primaquine, (−)primaquine, 5 hydroxyprimaquine (5HPQ), and 6-desmethyl-5-hydroxyprimaquine (6D5HPQ) were synthesized in the laboratory by one of us (R.C.G.). The optical rotation of (+)PQ was 30.75 and that of (−)PQ was 30.67. This compares well with values reported by

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Carroll *et al.* [12]. The metabolites were synthesized by selective demethylation of 5-methoxyprimaquine using 48% hydrobromic acid as described by Allahyari *et al.* [13]. The products showed a single peak on HPLC and have been characterized by spectroscopic analysis. The melting points of 5HPQ and 6D5HPQ were 209–211° and 206–209° respectively. The structural formulae of the compounds used in this study are given in our earlier publication [10].

Drug treatment and malonyldialdehyde (MDA) estimation. A 1-mL aliquot of whole blood in triplicate was incubated with either PQ, PQ enantiomers or its metabolites at a final concentration of 1.5 mM. This concentration is similar to that used in several other studies including our earlier study [10, 14–16]. To one set an equal volume of saline was added to serve as the control. Incubation was carried out at 37° in a water bath. After a 1-hr incubation, blood was centrifuged immediately at 1500 rpm for 10 min in a refrigerated centrifuge. The plasma and buffy coat were removed carefully leaving behind an erythrocyte pellet at the bottom of the tube. The pellet was washed three times with 2–3 vol. of phosphate-buffered saline (PBS) containing 4.0 mM sodium azide (to inhibit catalase). Packed red cells were suspended to a 20% hematocrit with buffered saline, and aliquots were taken for the estimation of MDA according to the method described by Sree Kumar *et al.* [17].

Merocyanine 540 staining. A modified procedure of Valinsky *et al.* [18] was followed. A 20-μL aliquot (10^8 cells/mL) was taken from the 1-hr incubated cell suspension of drug and non-drug-treated red cells. It was washed three times with buffered saline, pH 7.2 (0.14 M NaCl, 0.01 M KCl, 3 mM $MgCl_2$, 10 mM Tris). The cell pellet was reconstituted to 1 mL with low ionic strength incubation medium (LISIM) consisting of 0.25 mM sucrose, 15 mM sodium chloride, 5 mM KCl, 3 mM $MgCl_2$, 10 mM Tris supplemented with 5% bovine serum albumin and 1.75 μM Merocyanine (40 μL). Tubes were incubated at 37° for 30 min under complete darkness. Cells were washed three times with LISIM to get rid of unbound MC 540. Finally, cells were suspended in 100 μL of LISIM, loaded on a slide, and observed under an epifluorescence microscope (Zeiss) with a BP 450-490 excitation filter and an LP 520 barrier filter. Orange red fluorescent RBCs were recorded as a percentage of the total red cells.

Scanning electron microscopy (SEM). For scanning electron microscopy, 20-μL aliquots of suspension were taken and washed three times with 100 mM phosphate-buffered saline (pH 7.4) and centrifuged at 200 rpm for 10 min. The cells were resuspended in 1 mL of buffered saline and fixed with 0.25% glutaraldehyde for 30 min at room temperature. After fixation, the cells were washed three times with buffered saline and treated with osmium tetraoxide (1%) for 2 hr. The fixed cell suspension was washed and dehydrated with increased concentrations of alcohol. The stubs were prepared, coated with gold, and examined under the scanning electron microscope at 15 kV. Echinocytes and stomatocytes were recorded as a percentage of the total red cells.

RESULTS

Lipid peroxidation. Lipid peroxidation was measured by quantitation of MDA content. The data (Table 1) showed no significant difference in the basal MDA content in saline-treated normal, heterozygous and hemizygous G-6-PD deficient erythrocytes ($P > 0.05$). PQ and its enantiomers significantly increased MDA content in hemizygous G-6-PD deficient red cells ($P < 0.05$), but did not have any effect on normal red cells. PQ enantiomers did not show any differential effect ($P > 0.05$) in all three types of cells.

Unlike PQ, its hydroxy metabolites produced a significant increase in MDA content in both normal and heterozygous individuals as well as in G-6-PD deficient red cells ($P < 0.001$). Of the two hydroxy metabolites, 6D5HPQ was more toxic than 5HPQ at a 1.5 mM concentration in all categories of individuals studied.

Merocyanine (MC 540) staining. Alterations in membrane fluidity of red cells exposed to PQ, (+)PQ, (−)PQ and its metabolites were evaluated by using MC 540 as a fluorescent probe. No significant fluorescence was detected in saline-treated normal, heterozygous and hemizygous G6PD deficient red cells (Table 2). Upon incubation with PQ, (+)PQ and (−)PQ, fluorescence was seen in 10.5 ± 1.3, 11.7 ± 7.4, and 6.5 ± 2.6% of normal red cells; 26.5 ± 14.6, 58.8 ± 15.4 and 41.3 ± 20.1% of heterozygous red cells, and 83.0 ± 8.8, 84.8 ± 9.7 and 62.5 ± 26.8% of hemizygous G-6-PD deficient red cells.

Compared to PQ and its enantiomers, cells treated with the putative hydroxy metabolites of PQ showed significantly persistent and bright fluorescence on MC 540 staining ($P < 0.01$).

Fluorescence detected in drug-treated red cells was of two types: PQ, (+)PQ and (−)PQ treated red cells gave a speckled and fast quenching type of fluorescence, whereas 5HPQ and 6D5HPQ treated cells gave homogenous, comparatively bright, and long-lasting fluorescence. The intensity of fluorescence decreased in the following manner 6D5HPQ > 5HPQ > (+)PQ ≤ PQ > (−)PQ.

Scanning electron microscopy. The effects of PQ enantiomers and PQ metabolites on red cell surface morphology of normal, heterozygous and hemizygous G-6-PD deficient human erythrocytes as seen under a scanning electron microscope are shown in Fig. 1 and Table 3. The effect of PQ *per se* on this parameter was not studied.

(+)PQ treated red cells from healthy volunteers showed 42.0 ± 10.0% discoid shaped red cells with holes (stomatocytes), whereas in heterozygous and hemizygous G-6-PD deficient red cells the corresponding numbers were 61.5 ± 12.0 and 81.1 ± 11% respectively.

(−)PQ treated red cells showed changes in morphology similar to that of (+)PQ treated cells in normals (43.5 ± 7.8%). In heterozygous and hemizygous G6PD deficient red cells, 49.8 ± 15.2 and 93.3 ± 8.0% cells, respectively, became stomatocytic.

Unlike enantiomers, the putative metabolites of PQ led to echinocytic changes in all the individuals.

Table 1. Effects of primaquine, primaquine enantiomers and putative metabolites on malonyldialdehyde (MDA) content

Incubation medium	MDA content (nmol)		
	Normal (A)	Heterozygous (B)	Hemizygous deficient (C)
Saline	1.65 ± 0.55	1.69 ± 0.63	1.61 ± 0.61
PQ	1.60 ± 0.63	1.78 ± 0.08	2.69 ± 0.31
(+)PQ	1.73 ± 0.66	2.45 ± 0.24	2.45 ± 0.52
(−)PQ	1.90 ± 0.55	2.23 ± 0.24	2.64 ± 0.65
5HPQ	5.25 ± 0.31	5.59 ± 0.46	5.39 ± 0.24
6D5HPQ	6.53 ± 0.51	7.03 ± 0.24	6.64 ± 0.62

Values are means ± SD, N = 4. All drugs were tested at a 1.5 mM concentration. Statistical analysis: Normal saline vs PQ: No significant difference (NS) for A and B; and $P < 0.05$ for C; (+)PQ vs (−)PQ: NS for A, B and C; 5HPQ vs 6D5HPQ: $P < 0.05$ for A and < 0.01 for B and C; PQ vs hydroxymetabolites: $P < 0.001$ for A, B and C.

Table 2. Effects of primaquine, primaquine enantiomers and its putative metabolites on merocyanine 540 staining

Incubation medium	% Fluorescent cells		
	Normal (A)	Heterozygous (B)	Hemizygous deficient (C)
Saline	4.0 ± 1.8	7.5 ± 2.6	10.25 ± 7.8
PQ	10.5 ± 1.3	26.5 ± 14.6	83.0 + 8.8
(+)PQ	11.7 ± 7.4	58.8 ± 15.4	84.8 ± 9.7
(−)PQ	6.5 ± 2.6	41.3 ± 20.1	62.5 ± 26.8
5HPQ	29.5 ± 8.2	87.7 ± 8.2	87.8 ± 6.0
6D5HPQ	76.5 ± 19.8	90.0 ± 7.0	96.8 ± 2.9

Values are means ± SD, N = 4. All drugs were tested at a 1.5 mM concentration. Statistical analysis: Saline vs PQ: $P < 0.01$ for A, $P < 0.05$ for B, and $P < 0.001$ for C; (+)PQ vs (−)PQ: not significant (NS) for A, B and C; 5HPQ vs 6D5HPQ: $P < 0.01$ for A, NS for B, and $P < 0.05$ for C; Hydroxy metabolites vs PQ: $P < 0.01$ for A and B, and NS for C.

At a 1.5 mM concentration, 5HPQ produced 60 ± 10.7, 69.2 ± 13.7 and 99.2 ± 1.9% echinocytes in normal, heterozygous and hemizygous G-6-PD deficient red cells. Almost similar results were obtained with 6D5HPQ.

DISCUSSION

The studies reported by Baird *et al.* [1] and our own study [10] had suggested that the toxicity of PQ on G-6-PD deficient red cells may be mediated via cell membrane damage. To test this concept further, we studied the effects of PQ, its enantiomers [(+)PQ and (−)PQ], and putative hydroxy metabolites on three parameters of cell membrane damage, namely lipid peroxidation which leads to production of MDA [19], alteration in the fluidity of the cell membrane detected by staining with MC-450 [18, 19], and changes in the appearance of the red cell as seen on scanning electron microscopy [19]. All compounds tested produced a significant effect on red cell membranes. This effect was most marked in G-6-PD deficient red cells. Besides an increase in MDA content, a marked MC 450 fluorescence and change in membrane morphology were recorded. This indicates that alteration in the fluidity of the cell membrane is not totally dependent on unsaturated fatty acid content of the cell membrane. Lipid asymmetry has been considered to be a function of lipid–protein interaction [20, 21]. PQ may cause oxidation of membrane proteins resulting in formation of S—S bonds between SH groups of membrane or aggregation of spectrin. The latter is known to cause an alteration in transbilayer asymmetry leading to increased affinity for MC-540 [20]. The morphological changes in cell membrane caused by PQ enantiomers and its hydroxy metabolites, as seen by scanning electron microscopy in this study, lend further support to the hypothesis that the cell membrane is the target of PQ toxicity.

Our previous study showed discordant behavior of the two enantiomers of PQ on intracellular oxidation and plasma membrane leakiness [10]. While (−)PQ produced a significantly greater effect on GSH and methemoglobin levels, (+)PQ was found to produce significantly greater membrane leakiness compared to (−)PQ. The present study has shown that (+)PQ produced more membrane changes in red cells than

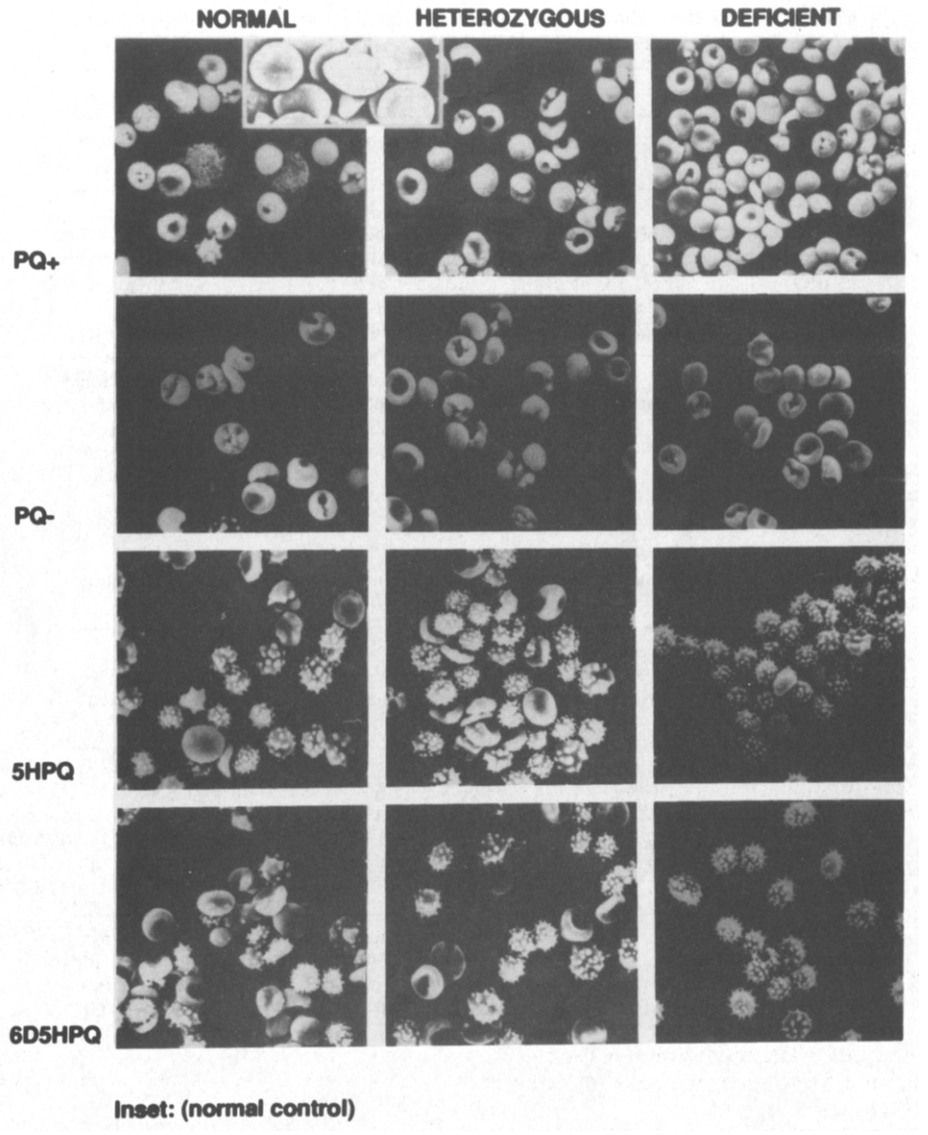

Fig. 1. Effects of PQ enantiomers and PQ metabolites on red cell surface morphology of normal, heterozygous and hemizygous G-6-PD deficient human erythrocytes (×1500). Inset: Saline-treated normal cells (×3000).

Table 3. Scanning electron microscopic studies of RBC after treatment with primaquine enantiomers and its putative metabolites

	% Cells showing abnormal appearance		
	Normal (A)	Heterozygous (B)	Hemizygous deficient (C)
(+)PQ	42.0 ± 10.0	61.5 ± 12.0	81.1 ± 11.0
(−)PQ	43.5 ± 7.8	49.8 ± 15.2	93.3 ± 8.0
5HPQ	60.0 ± 10.7	69.2 ± 13.7	99.2 ± 1.9
6D5HPQ	61.5 ± 11.7	78.0 ± 10.5	95.8 ± 4.1

Values are means ± SD, N = 10. All drugs were tested at a 1.5 mM concentration. Statistical analysis: (+)PQ vs (−)PQ: NS for A and B, and $P < 0.05$ for C; 5HPQ vs 6D5HPQ: $P > 0.05$ for A and B, and $P < 0.05$ for C; Enantiomers vs metabolites: $P < 0.01$ for A and B, and $P < 0.05$ for C.

(−)PQ (Table 3). However, the effects on MDA content and percentage fluorescence were not different for the two enantiomers (Tables 1 and 2). It could have been the function of the concentration of the compounds tested in this study.

The hydroxy metabolites of PQ were significantly more toxic to red cell membranes than were PQ and its enantiomers. These compounds increased the content of MDA, enhanced staining with MC-540, and led to marked changes on SEM in a significant proportion of the cells. Again this may be a consequence of the concentration used in this study (1.5 mM) which was kept equimolar to that of PQ and its enantiomers for the sake of comparison. The difference in degree of toxicity of the two hydroxy metabolites was marginal, but 6D5HPQ was slightly more toxic than 5HPQ.

PQ enantiomers had a stomatocytic effect on RBCs, whereas the putative metabolites of PQ had an echinocytic effect. It is conceivable that, analogous to albumin, PQ may have affinity for lysolecithin. After incubation it may cause extraction of lysolecithin from the outer hemileaflet but not from the inner one, causing stomatocytic transformation according to the bilayer couple theory. According to Reinhart and Chien [22], spherostomatocytes are capable of ecinocytic transformation (with specule formation within the red cell vacuoles), whereas spheroechinocytes are unable to undergo stomatocytic transformation without hemolysis. It is possible that, initially, putative metabolites of PQ may also induce the formation of stomatocytes which rapidly transform into echinocytes.

The sensitivity of the MC-450 staining test appears to be similar to that of SEM studies. At the concentration tested, (+)PQ and (−)PQ produced fluorescence in 11.7 ± 7.4 and 6.5 ± 2.6% of normal red cells respectively ($P > 0.05$) and changes on SEM were seen in 42.0 ± 10.0 and 43.5 ± 7.8% of normal red cells ($P > 0.05$). The relationship between MC-450 staining and SEM changes needs to be investigated further.

Evidence presented thus far suggests that PQ toxicity on red cell membrane proteins may be mediated through its effect on membrane lipid and oxidation of intracellular contents. These represent, however, indirect evidence. Investigations are needed to elucidate precisely the primary event in toxicity so that safer analogues of PQ can be developed and also suitable methods for toxicity prevention devised.

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