Primaquine-Induced Hemolytic Anemia: Effect of 6-Methoxy-8-hydroxylaminoquinoline on Rat Erythrocyte Sulfhydryl Status, Membrane Lipids, Cytoskeletal Proteins, and Morphology

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ABSTRACT

Previous studies have shown that 6-methoxy-8-hydroxylaminoquinoline (MAQ-NOH), an N-hydroxy metabolite of the antimalarial drug, primaquine, is a direct-acting hemolytic agent in rats. To investigate the mechanism underlying this hemolytic activity, the effects of hemotoxic concentrations of MAQ-NOH on rat erythrocyte sulfhydryl status, membrane lipids, skeletal proteins, and morphology have been examined. Treatment of rat erythrocytes with a TC50 concentration of MAQ-NOH (350 μM) caused only a modest and transient depletion of reduced glutathione (GSH) (~30%), which was matched by modest increases in the levels of glutathione disulfide and glutathione-protein mixed disulfides. Lipid peroxidation, as measured by thiobarbituric acid-reactive substances and F2-isoprostane formation, was induced in a concentration-dependent manner by MAQ-NOH. However, the formation of disulfide-linked hemoglobin adducts on membrane skeletal proteins and changes in erythrocyte morphology were not observed. These data suggest that hemolytic activity results from peroxidative damage to the lipid of the red cell membrane and is not dependent on skeletal protein thiol oxidation. However, when red cell GSH was depleted (>90%) by titration with diethyl maleate, hemolytic activity of MAQ-NOH was markedly enhanced. Of interest, exacerbation of hemotoxicity was not matched by increases in lipid peroxidation, but by the appearance of hemoglobin-skeletal protein adducts. Collectively, the data are consistent with the concept that MAQ-NOH may operate by more than one mechanism; one that involves lipid peroxidation in the presence of normal amounts of erythrocytic GSH, and one that involves protein oxidation in red cells with low levels of GSH, such as are seen in individuals with glucose-6-phosphate dehydrogenase deficiency.
The hemotoxic metabolite, 6-methoxy-8-aminooquinoline, can be N-hydroxylated to form 6-methoxy-8-hydroxyaminooquinoline (MAQ-NOH) by both rat and human microsomes (Bolchoz et al., 2001). Furthermore, MAQ-NOH was found to be a direct-acting hemolytic agent in the rat; that is, when rat $^{51}$Cr-labeled red cells are incubated with MAQ-NOH in vitro and returned to isologous rats, the labeled red cells are more rapidly removed from the circulation than are the vehicle-treated control cells.

Although sequestration of senescent or damaged (but intact) red cells from the circulation is known to occur by macrophages of the reticuloendothelial system (Rifkind, 1966), the signal that marks these cells for removal remains unknown and continues to be a subject for debate (for review, see Bratosin et al., 1998). One group of studies suggests that peroxidative damage to the plasma membrane lipid bilayer transmits the signal for sequestration (Lubin and Chiu, 1982; Zwaal and Schroit, 1997). Alternatively, membrane skeletal protein alterations may underlie the removal process. For example, alteration in the lateral distribution of band 3 protein via hemoglobin (or hemichrome) binding has been shown to result in the binding of autologous antibodies, which commit the cells for uptake by cultured monocytes (Waugh et al., 1987; Turrini et al., 1991).

In view of the critical role proposed for oxidant damage in the mechanism underlying primaquine-induced hemolytic anemia, we have examined the effect of MAQ-NOH on sulfhydryl status, membrane lipids, and skeletal proteins in suspensions of rat erythrocytes using MAQ-NOH concentrations known to induce the premature removal of these cells from the circulation. We report that MAQ-NOH has only a modest capacity to oxidize red cell GSH to glutathione disulfide (GSSG) and glutathione-protein (GS-protein) mixed disulfides, and to induce the formation of hemoglobin-skeletal protein adducts. In contrast to other arylhydroxylamines, exposure of normal rat red cells to MAQ-NOH significantly enhanced the peroxidation of membrane lipids. When GSH was depleted from rat red cells to mimic the low levels of GSH that are observed in the human G6PD-deficient red cell (Gaetani et al., 1979), the hemolytic activity of MAQ-NOH was markedly enhanced. This exacerbation was associated with the development of protein thiol oxidation without change in the level of lipid peroxidation. It is suggested that MAQ-NOH can inflict hemolytic injury on the red cell by two pathways: lipid peroxidation in GSH-normal red cells and protein oxidation in GSH-depleted red cells. The possibility of synergistic interaction between these processes and among the hemotoxic metabolites of primaquine is discussed.

**Materials and Methods**

**Chemicals and Materials.** MAQ-NOH was synthesized as described previously (Allahyari et al., 1984). Malondialdehyde, diethyl maleate (DEM), and rabbit anti-rat hemoglobin IgG were purchased from Sigma-Aldrich (St Louis, MO). Horseradish peroxidase-conjugated donkey anti-rabbit IgG was purchased from Amersham Biosciences Inc. (Piscataway, NJ). Na$_2$^{14}CrO$_4$ in sterile saline (1 mCi/ml, pH 8) was obtained from PerkinElmer Life Sciences (Billerica, MA). All other chemicals were of the best commercially available grade.

**Animals.** Male Sprague-Dawley rats (75–100 g) were purchased from Harlan (Indianapolis, IN) and were maintained on food and water ad libitum. Animals were acclimated to a 12-h light/dark cycle prior to their use.

**Red Cell Incubation Conditions.** Blood from the descending aorta of anesthetized rats was collected into heparinized tubes and washed in isotonic phosphate-buffered saline (pH 7.4) supplemented with 10 mM d-glucose (PBSG). After removal of the plasma, the red cells were resuspended in PBSG to a 40% hematocrit and used the same day they were collected. Experiments were carried out by addition of various concentrations of MAQ-NOH dissolved in DMSO (10 μl) to the erythrocyte suspensions (2 ml) and allowed to incubate at 37°C for up to 2 h.

**Determination of Sulphydryl Status.** Aliquots (0.2 ml) of the incubation mixtures were removed at various intervals after the addition of MAQ-NOH and assayed for GSH, GSSG, and GS-protein mixed disulfides by HPLC with electrochemical detection as described previously (Grossman et al., 1992). The amount of sulphydryl present in the samples was determined by comparison of peak heights to standards prepared identically to the samples.

**Morphological Examination of Red Cells.** After incubation, red cells treated with the vehicle (DMSO) or MAQ-NOH were washed and prepared for scanning electron microscopy as previously described (Grossman et al., 1992). Briefly, the cells were fixed in 1% glutaraldehyde, 2% formalin, and 0.1 M cacodylate buffer. The cells were affixed onto 0.2-μm filters and dehydrated in ethanol. The cells were then dried by critical point drying, cast with a thin coat of carbon and gold, and examined in a JEOL (Tokyo, Japan) JSM-5410LV scanning electron microscope operating at a 10-kV accelerating voltage.

**Determination of Lipid Peroxidation in Red Cells.** Following a 60-min incubation in the presence of MAQ-NOH, red cells were analyzed for thiobarbituric acid-reactive substance (TBARS) as previously described (McMillan et al., 1998). TBARS was quantitated based on a standard curve generated with known amounts of malondialdehyde. Lipid peroxidation was also assessed by measuring the level of F$_2$-isoprostanes in red cell ghosts prepared from MAQ-NOH-treated red cells by gas chromatography-mass spectrometry analysis as described previously (Morrow and Roberts, 1999).

**Electrophoretic Analysis of Membrane Skeletal Proteins.** Red cell ghosts were prepared from vehicle- and MAQ-NOH-treated red cells as described previously (Grossman et al., 1992). Red cell ghosts were also prepared from red cells treated with dapsone hydroxylamine and were used as a positive control for the presence of hemoglobin-skeletal protein adducts (Grossman et al., 1992). The ghosts were washed extensively to remove any unbound hemoglobin and were solubilized in SDS. Electrophoretic and immunoblotting analysis of the solubilized ghosts was carried out as previously described (McMillan et al., 1995). The skeletal proteins were resolved on nonreducing, continuous gels consisting of 5% monomer and 1.5% bis-acrylamide crosslinker. Protein bands were identified according to their migration distance (Fairbanks et al., 1971).

**GSH Depletion of Red Cell Suspensions.** DEM (750 μM) dissolved in acetone was added to packed red cells at an initial concentration of 0.121 μM of packed cells. After a 15-min incubation at 37°C, the red cells were analyzed for GSH content by HPLC with electrochemical detection as described above. This treatment typically reduced GSH concentrations to about 5 to 10% of the initial level, and this level of depletion was maintained throughout the course of the experiment. The cells were then resuspended in PBSG (40% suspension) and used on the same day that they were collected.

**Determination of the Hemolytic Response.** The survival of $^{51}$Cr-labeled red cells in vivo after in vitro exposure to MAQ-NOH was determined as described previously (Harrison and Jollow, 1986). After the incubation, the red cells were washed and resuspended (40% hematocrit), and an aliquot (0.5 ml) was administered i.v. to isologous rats. T$_0$ blood samples were taken from the orbital sinus 30 min after the administration of the labeled cells. Additional samples were taken in 48-h intervals for 14 days. At the end of the experiment the samples were counted in a well-type gamma counter, and the data were expressed as a percentage of the T$_0$ blood sample.
Statistical significance was determined with the use of Student’s t test.

**Results**

**Effect of MAQ-NOH on Rat Erythrocyte Sulfhydryl Status.** Instability of red cell GSH has long been considered as a hallmark of “primaquine sensitivity”; that is, that the administration of primaquine to G6PD-deficient individuals results in a fall in the GSH level in their circulating red cells (Tarlov et al., 1962; Beutler, 1969). Thus, we examined the effect MAQ-NOH on GSH levels in rat erythrocytes in vitro under previously established hemolytic conditions (Bolchoz et al., 2001). MAQ-NOH was added to rat red cell suspensions, and aliquots were taken at various time points and analyzed for GSH, GSSG, and GS-protein mixed disulfides by HPLC. As shown in Fig. 1A, addition of a TC50 concentration of MAQ-NOH (350 μM) to rat red cells resulted in a transient decline of GSH to about 70% of initial levels, reaching a nadir within 30 min. The loss of GSH was matched by an increase in both GSSG and GS-protein mixed disulfides.

Increasing concentrations of MAQ-NOH showed the same general pattern of response: rapid but transient loss of GSH with a nadir at about 30 min, with a corresponding formation of GSSG and GS-protein. The concentration dependence of MAQ-NOH-induced depletion of red cell GSH levels was examined by plotting the nadir of GSH content (at 30 min) against MAQ-NOH concentration. As shown in Fig. 1B, the EC50 of GSH depletion by MAQ-NOH was about 1000 μM. Of interest, this concentration-response curve for MAQ-NOH-induced GSH oxidation is shifted significantly to the right of the concentration-response curve previously reported for MAQ-NOH hemolytic activity (TC50 ca. 350 μM) (Bolchoz et al., 2001).

**Effect of MAQ-NOH on Rat Erythrocyte Membrane Lipids.** To determine whether lipid peroxidation could be detected in rat red cell suspensions exposed to MAQ-NOH, TBARS formation was measured after a 60 min incubation with MAQ-NOH. As shown in Fig. 2A, the amount of TBARS formed in MAQ-NOH-treated red cells was increased significantly as compared with the vehicle-treated control cells.

**Fig. 1.** A, effect of MAQ-NOH on rat erythrocyte sulfhydryl status. Rat erythrocytes were incubated at 37°C in PBSG containing MAQ-NOH (350 μM). At the indicated time points, aliquots were withdrawn and assayed for GSH, GSSG, and protein-glutathione mixed disulfides. The values shown are means ± S.D. (n = 3). B, concentration-response relationship for GSH oxidation by MAQ-NOH in rat erythrocytes. Percentage of reduction in GSH was determined using the nadir values for GSH as compared with controls. Values are means of duplicate determinations.

**Fig. 2.** A, formation of TBARS in rat erythrocytes exposed in vitro to MAQ-NOH. Rat erythrocytes were incubated at 37°C for 60 min in PBSG containing the indicated concentrations of MAQ-NOH. Control cells were incubated with vehicle alone (10 μl of DMSO). After the incubation, the cells were lysed, centrifuged, and analyzed for TBARS, as described under Materials and Methods. The values are means ± S.D. (p < 0.05, n = 5). B, formation of F2-isoprostanes in ghosts prepared from rat erythrocytes exposed in vitro to MAQ-NOH. Erythrocyte suspensions (40% hematocrit) were incubated with the indicated concentrations of MAQ-NOH for 2 h at 37°C. After incubation, erythrocyte ghosts were prepared and analyzed for F2-isoprostane content, as described under Materials and Methods. The values are means ± S.D. (n = 4). *, significantly different from control (p < 0.05).

TBARS formation was dependent on MAQ-NOH concentration up to 350 μM.

Although TBARS is a widely accepted method for the measurement of lipid peroxidation, problems with the specificity of this assay are well known (Moore and Roberts, 1998).
Thus, F$_2$-isoprostane content in MAQ-NOH-treated red cell ghosts was used to confirm the induction of lipid peroxidation. Following a 2-h incubation with various concentrations of MAQ-NOH, the cells were washed and lysed in hypotonic saline to prepare red cell ghosts. Esterified isoprostanes were hydrolyzed and extracted, and the content of free F$_2$-isoprostanes in the sample was quantified by gas chromatography-mass spectrometry. As shown in Fig. 2B, MAQ-NOH induced a concentration-dependent increase in the formation of F$_2$-isoprostanes, which confirmed the results obtained with the TBARS assay.

**Effect of MAQ-NOH on Rat Erythrocyte Membrane Skeletal Proteins.** We have shown previously that the hemolytic response to the N-hydroxy metabolite of the arylamine drug dapsone is associated with profound oxidative damage to the membrane cytoskeleton (Grossman et al., 1992; McMillan et al., 1995). The damage was found to be the result of formation of disulfide-linked hemoglobin adducts on certain skeletal protein bands and the appearance of membrane-bound hemoglobin aggregates, and these alterations were accompanied by changes in the shape of the red cells to a severe (type III) echinocytic morphology. To determine whether hemolytic concentrations of MAQ-NOH could induce similar changes in the cytoskeletal proteins, membrane skeletal proteins from control, dapsone hydroxylamine (included as a positive control), and MAQ-NOH-treated red cells were separated on SDS-polyacrylamide gels and either stained with Gel Code Blue or transferred to polyvinylidene difluoride membranes and immunostained with anti-hemoglobin polyclonal antibodies. As shown in Fig. 3A, the electrophoretic pattern of membrane skeletal proteins of the positive control (dapsone hydroxylamine, lanes 6–7) showed the characteristic changes previously described (Grossman et al., 1992); viz., broadening of protein bands 1 and 2, loss of bands 2.1 and 4.2, splitting of band 3, and formation of hemoglobin aggregates. Immunoblot analysis using the anti-hemoglobin antibody (Fig. 3B, lanes 6–7) confirmed that the changes in electrophoretic mobility were due to formation of hemoglobin adducts with the skeletal proteins, and of the presence of monomeric and polymeric forms of hemoglobin.

MAQ-NOH-treated red cells (Fig. 3A, lanes 2–5) were not significantly different from the control (lane 1) in regard to the mobility of the skeletal proteins. The absence of significant levels of skeletal protein-hemoglobin adducts was confirmed by the anti-hemoglobin immunoblot (Fig. 3B, lanes 2–5). Of interest, a concentration-dependent increase in the amount of hemoglobin monomer (16 kDa) was observed in

![Fig. 3. Effect of MAQ-NOH on rat erythrocyte membrane skeletal proteins.](image)

![Fig. 4. Effect of MAQ-NOH on rat erythrocyte morphology.](image)
the Gel Code Blue-stained gel, as well as in the anti-hemoglobin immunoblot (Fig. 3B, lanes 2–5).

**Effect of MAQ-NOH on Rat Erythrocyte Morphology.**
To investigate the effect of MAQ-NOH on rat erythrocyte morphology, red cell suspensions were incubated in the presence and absence of MAQ-NOH, and aliquots of the cells were prepared for scanning electron microscopy. As shown in Fig. 4A, red cells treated with the vehicle (10 μl of DMSO in 2 ml of red cell suspension) for 2 h at 37°C retained their normal biconcave appearance, although occasional small protuberances were observed in some of the cells. These protuberances were not seen in cells incubated in normal saline alone and are attributed to the DMSO. Rat erythrocytes incubated with MAQ-NOH, even at a very high concentration (1 mM; Fig. 4B), also exhibited normal discocytic morphology.

**Effect of MAQ-NOH on GSH-Depleted Erythrocytes.**
The enhanced sensitivity of red cells from G6PD-deficient individuals to the oxidative actions of certain drugs is considered to be due to their diminished capacity to maintain sufficient levels of NADPH, and hence GSH, when challenged by the oxidant stress. In an effort to reproduce this enhanced sensitivity in normal rat red cells, GSH was depleted by >90% using DEM pretreatment as described under Materials and Methods.

**TABLE 1**
Effect of GSH depletion on MAQ-NOH-induced TBARS formation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TBARS (μM)</th>
<th>Mean ± S.D. (n = 3)</th>
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<tbody>
<tr>
<td>Control</td>
<td>0.15 ± 0.18</td>
<td></td>
</tr>
<tr>
<td>MAQ-NOH (250 μM)</td>
<td>0.35 ± 0.11</td>
<td></td>
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<tr>
<td>MAQ-NOH (–GSH)&lt;b&gt;</td>
<td>0.25 ± 0.15</td>
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* Significantly different from the control (p < 0.05).

b Red cell GSH was depleted by >90% of normal levels using DEM pretreatment as described under Materials and Methods.

**Fig. 5.** The survival of GSH-depleted 51Cr-labeled erythrocytes in vivo after in vitro exposure to 250 μM MAQ-NOH. Radiolabeled red cells were treated with DEM to deplete intracellular GSH (>90%). The cells were then incubated with vehicle or 250 μM MAQ-NOH for 2 h at 37°C. The erythrocytes were then washed and administered i.v. into a group of isologous rats. T<sub>50</sub> blood samples were taken 30 min after administration of the labeled cells. Data points are means ± S.D. (n = 4).

**Fig. 6.** Effect of MAQ-NOH on the membrane skeletal proteins of GSH-depleted erythrocytes. GSH depleted (90%) rat erythrocytes were incubated in the presence of DMSO (lane 1), 150 μM MAQ-NOH (lane 2), 250 μM MAQ-NOH (lane 3), or 350 μM MAQ-NOH (lane 4) for 2 h at 37°C. Following exposure, the cells were washed, and membrane ghosts were prepared and washed extensively to remove unbound hemoglobin. The ghosts (32 μg of protein) were solubilized in SDS and subjected to PAGE. A, Gel Code Blue-stained gel and densitometric scans; B, immunoblot stained with rabbit anti-rat hemoglobin.

**TABLE 1**
Effect of GSH depletion on MAQ-NOH-induced TBARS formation

Rat erythrocyte suspensions (2 ml, 40% hematocrit in PBSG) were incubated at 37°C for 60 min with the vehicle (20 μl of DMSO) or MAQ-NOH (250 μM). The values shown are means ± S.D. (n = 3).

<table>
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<tr>
<th>Treatment</th>
<th>TBARS (μM)</th>
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* Significantly different from the control (p < 0.05).

b Red cell GSH was depleted by >90% of normal levels using DEM pretreatment as described under Materials and Methods.
oxidation was detected in MAQ-NOH-treated red cells using concentrations of DDS-NOH (McMillan et al., 1998). Lipid per-observed previously in red cells treated with hemolytic con-
trations tested, the depletion of cellular GSH per se is not a cause depletion of red cell GSH within the range of concen-
This relationship suggests that although MAQ-NOH does not visibly change in MAQ-NOH-treated, GSH-defi-
cells to spheroechinocytes (Rifkind and Danon, 1965).

Discussion

The present results demonstrate that under in vitro incubation conditions known to commit rat erythrocytes to pre-
mature removal from the circulation after their re-adminis-
tration to isologous animals, MAQ-NOH induced alterations in red cell sulfhydryl status (Fig. 1) and caused damage to the plasma membrane in the form of lipid peroxidation (Fig. 2). Overall, the data presented here support the concept that oxidative stress underlies MAQ-NOH hemolytic activity.

On the one hand, the ability of MAQ-NOH to deplete GSH was not unexpected in view of our previous studies with other arylhydroxylamines, and as with these other agents, the loss of GSH could be accounted for by oxidation to GSSG and GS-protein. However, examination of the time course (Fig. 1A) and concentration dependence (Fig. 1B) for MAQ-NOH-induced alterations in sulfhydryl status indicated that there are striking differences between this compound and previously examined N-hydroxy compounds. For example, with DDS-NOH, hemolytic activity is associated with a rapid, extensive, and long-lasting depletion of red cell GSH (Grossman et al., 1992). In the case of MAQ-NOH, the loss of GSH was transient and relatively modest across the full range of hemolytic concentrations. These data indicate that the MAQ-NOH concentration-response curve for GSH oxidation (EC$_{50}$ ca. 1000 μM) is well to the right of the curve for the hemolytic response, which has a TC$_{50}$ of about 350 μM (Bolchoz et al., 2001). This relationship suggests that although MAQ-NOH does cause depletion of red cell GSH within the range of concent-
trations tested, the depletion of cellular GSH per se is not a prerequisite for the oxidative stress-mediated hemolytic damage that MAQ-NOH inflicts on the red cell.

On the other hand, MAQ-NOH-induced lipid peroxidation was unexpected in view of the lack of lipid peroxidation observed previously in red cells treated with hemolytic concentrations of DDS-NOH (McMillan et al., 1998). Lipid peroxidation was detected in MAQ-NOH-treated red cells using two independent measurements, TBARS and F$_2$-isoprostanes (Fig. 2), and occurred across the range of hemolytic concentrations of MAQ-NOH. In contrast, cytoskeletal protein thiol oxidation, as indicated by formation of hemoglobin-skel-
tetal protein adducts, was not significant after exposure of red cells to hemolytic concentrations of MAQ-NOH (Fig. 3). These data suggested that skeletal protein is not the pre-
ferred intracellular target of MAQ-NOH, as it is for DDS-NOH.

It should be noted that although skeletal protein was ap-
parently not affected by MAQ-NOH, there was a concentra-
tion-dependent increase in the amount of hemoglobin mono-
mer present in membrane ghosts prepared from MAQ-NOH-
treated red cells (Fig. 3). This monomer could not be removed from the ghosts by repetitive washing and is presumed to be bound either covalently (hydrophobic interaction) or co-
valently to the lipid matrix. The molecular form of this mem-
brane-bound hemoglobin and its significance remain to be determined. Nevertheless, binding of the monomer may be a key event in the hemolytic process because it is observed with other arylhydroxylamines, such as DDS-NOH (Grossman et al., 1992), and has been seen with other hemolytic agents, such as divicine (McMillan et al., 2001), as well as in rat red cells aged to senescence by the serial hypertransfusion tech-
nique (D. C. McMillan and D. J. Jollow, unpublished obser-

Alterations in erythrocyte morphology were notably absent in MAQ-NOH-treated red cells (Fig. 4). In view of the well known role of the skeletal protein assembly in the mainte-
nance of red cell shape (Marchesi, 1985), the lack of gross morphological changes in these red cells is consistent with the absence of changes in the electrophoretic mobility of the skeletal proteins on SDS-polyacrylamide gels (Figs. 3 and 6). The lack of morphological change after hemolytic concentra-
tions of MAQ-NOH is in sharp contrast to the dramatic changes in erythrocyte morphology observed with other he-
molytic agents. For example, hemolytic concentrations of DDS-NOH and divicine induce distinctive echinocytic mor-
phology (Grossman et al., 1992; McMillan et al., 2001); and phenylhydrazine is well known to cause transformation of red cells to spherocytocyes (Rifkind and Danon, 1965).

Although the data presented here support an oxidative stress-type mechanism for MAQ-NOH hemotoxicity, its low potency as a GSH-depleting agent made us wonder whether cells lacking GSH (or the ability to replenish it) would be more sensitive to MAQ-NOH-induced oxidant damage. Fur-
thermore, we reasoned that since lipid peroxidation appeared to be causal in the hemolytic process, whereas cytoskeletal protein oxidation was not, then any enhancement in hemo-
toxicity provoked by depletion of GSH should be accompanied by corresponding increases in lipid peroxidation without af-
fected the level of protein oxidation. When red cell GSH was deple-
ted (>90%) by titration with DEM prior to exposure to MAQ-NOH, the hemolytic activity of MAQ-NOH was mark-
edly enhanced (Fig. 5). Surprisingly, however, exacerbation of hemolytic activity was not accompanied by an increase in lipid peroxidation (Table 1).

Although the SDS-PAGE pattern of the skeletal proteins was not visibly changed in MAQ-NOH-treated, GSH-defi-
cient red cells (Fig. 6A), examination of the skeletal proteins by immunoblotting revealed the presence of hemoglobin-skel-
tetal protein adducts. Moreover, the presence of these adducts in GSH-deficient red cells was dependent on MAQ-NOH con-
centration (Fig. 6B). The fact that the amount of hemoglobin adducted to the skeletal protein was insufficient to perturb the SDS-PAGE pattern suggests that it is quantitatively much less than that seen with equally hemotoxic concentra-
tions of a “pure” protein thiol oxidizer, such as DDS-NOH. This raises the possibility that protein oxidation and lipid peroxidation act additively, and perhaps even synergisti-
cally, in initiating the intracellular events that lead to pre-
mature splenic sequestration. However, it should be noted that the quantitative relationship between the initial “hit” and commitment of the cells to removal is not yet known. The possibility that MAQ-NOH may operate by multiple mecha-
nisms in the GSH-depleted red cell, and that these mechanisms may be synergistic, is intriguing and warrants further investigation.

We have shown previously that N-hydroxylamines are responsible for the hemolytic activity observed after administration of several arylamine drugs and environmental chemicals, including aniline (Harrison and Jollow, 1986), dapsone (Grossman and Jollow, 1988), phenacetin (Jensen and Jollow, 1991), and propanil (McMillan et al., 1991). Although the mechanism by which these N-hydroxylamines cause hemolytic injury is not completely understood, evidence suggests that these compounds produce damage within red cells as a consequence of their coupled oxidation with oxyhemoglobin, yielding methemoglobin and the arynitroso derivative. In the course of this reaction, reactive oxygen species, thyl radicals, and possibly other (i.e., compound-centered) free radicals are generated (Kiese, 1974; Maples et al., 1990; Bradshaw et al., 1997). It is believed that these free radicals oxidize critical sites within the red cell that ultimately transmit a signal to the external cell surface, marking the cell for removal from circulation by macrophages. The precise intracellular lesion responsible for premature sequestration of these cells, whether on lipid or protein, is still not known.

One theory is that free radical-induced membrane lipid peroxidation may play a role in transmitting a signal for removal. Jain and colleagues (see Jain, 1984) have shown that the lipid peroxidation byproduct maltoldehyde can flip phosphatidylserine from the inner to the outer leaflet of the plasma membrane. Disruption of the asymmetrical distribution of phosphatidylserine in the plasma membrane has been shown to stimulate erythrophagocytosis (Tanaka and Schroit, 1983; McEvoy et al., 1986; Bonomini et al., 2001). A second postulate suggests that sulfide-induced hemoglobin adds to critical membrane skeletal proteins, formed via hemoglobin thyl radical attack of the protein free sulphydryl groups, initiates red cell sequestration (Jollow et al., 1995; Jollow and McMillan, 1998). In support of this postulate, a number of investigators have shown that hemoglobin attachment to the cytosolic domain of band 3 causes alterations in the lateral distribution of this integral membrane protein leading to the binding of autologous antibodies on the external cell surface, which initiates erythrophagocytosis (Lutz et al., 1984; Waugh et al., 1986, 1997; Turrini et al., 1991).

Assessment of the contribution that MAQ-NOH makes toward the hemotoxicity of primaquine is difficult. One problem is that despite its use for over 50 years, the metabolism of primaquine remains poorly defined, and there are a multiplicity of unstable, redox-active metabolites that can potentially be formed during its metabolic clearance. These include several phenolic derivatives that have been implicated by others in the hemotoxicity of primaquine (Link et al., 1985; Augusto et al., 1988; Fletcher et al., 1988; Agarwal et al., 1991). Thus, it is conceivable that primaquine hemotoxicity in humans is mediated by more than one type of toxic metabolite.

Second, there may be interactions among these metabolites that facilitate the development of oxidative damage within the erythrocyte. For example, preliminary observations in our laboratory indicate that a phenolic metabolite, 5-hydroxyprimaquine, is also a direct-acting hemolytic agent. Furthermore, in contrast to MAQ-NOH, 5-hydroxyprimaquine is also a potent GSH-depleting agent (D. C. McMillan, D. J. Jollow, unpublished studies). This observation suggests that by depleting GSH, 5-hydroxyprimaquine may sensitize the red cell to the hemolytic activity of MAQ-NOH. If so, this effect would be amplified in G6PD-deficient red cells due to the instability of their erythrocrtic GSH. As noted by De-govin et al. (1966), G6PD-deficient individuals are about 20- to 30-fold more sensitive to the hemolytic activity of primaquine than are G6PD-normal individuals. Clearly, the possibility of metabolite synergism in primaquine-induced hemolytic anemia warrants further investigation.

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Kidney Dis 26:93-100.


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