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METABOLIC AND MORPHOLOGIC EFFECTS OF THE ANTIMICROBIAL AGENT NITROFURANTOIN ON HUMAN ERYTHROCYTES *IN VITRO**

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Abstract—We have reported previously that the antimicrobial nitrofurantoin stimulates superoxide production and methemoglobin formation from HbO_2 as an isolated hemeprotein and in hemolysates [M. Dershwitz and R. F. Novak, *J. biol. Chem.* **257**, 75 (1982); M. Dershwitz and R. F. Novak, *J. Pharmac. exp. Ther.* **222**, 430 (1982)]. The production of hydrogen peroxide and methemoglobin by nitrofurantoin has been determined in normal erythrocytes *in vitro*. Hydrogen peroxide production increased 5-fold during a 20-hr incubation in the presence of 840 μM nitrofurantoin, while methemoglobin content increased to over 20% of the total hemoglobin concentration of the cells. Consequent metabolic and morphologic alterations also occurred. Concomitant with nitrofurantoin-stimulated hydrogen peroxide production were time- and concentration-dependent decreases in cellular levels of GSH and ATP, as well as alterations in red cell morphology. Significant differences in GSH and ATP levels between control and nitrofurantoin-treated erythrocytes occurred after 12 hr and proceeded maximally from 18 to 21 hr. After a 21-hr incubation, 840 μM nitrofurantoin caused the cellular GSH and ATP levels to fall 65 and 75%, respectively, while controls exhibited only 29 and 43% decreases in ATP and GSH levels, respectively. Studies on the concentration dependence of such decreases demonstrated that the EC_{50} values for depletion of GSH and ATP were similar in blood obtained from an individual donor. The EC_{50} values varied from approximately 10 μM to 100 μM among the various donors whose blood was studied. Incubation of normal red cells with nitrofurantoin also resulted in an increased conversion of red cells to echinocytes as observed by scanning electron microscopy. These metabolic effects, coupled with increased oxidative stress via hydrogen peroxide generation, lend support to the mechanism for nitrofurantoin-induced hemolysis in erythrocytes compromised by certain enzyme deficiencies which result in low basal levels of GSH or diminished rates of GSH synthesis.

The antimicrobial nitrofurantoin (NF§) frequently causes red cell hemolysis when administered to persons with erythrocytes deficient in enzymes such as glucose-6-phosphate dehydrogenase (G-6-PD), glutathione peroxidase, glutathione reductase, phosphogluconic dehydrogenase and the enzymes active in glutathione synthesis [1]. These enzymes are responsible for the maintenance of adequate levels of reduced glutathione within the red cell. GSH is of significant importance in the red cell for several reasons: it serves as an important source of reducing equivalents in the conversion of hydrogen peroxide to water via glutathione peroxidase, and it acts as a scavenger of free radical species such as superoxide anion and certain reactive drug metabolites. It follows, therefore, that a drug-induced increase in the rate of superoxide generation or metabolic conversion of a drug to a reactive metabolite would result in the decrease of red cell GSH. Thus, the red cell hemolysis produced by nitrofurantoin may well involve the increased production of reactive chemical species ($O_2^{\cdot}/H_2O_2$; nitroaromatic anion free radical) dependent upon GSH for detoxification, with these species thereby serving to markedly deplete GSH levels while causing a concomitant increase in energy demand (i.e. ATP, NADPH) and alterations in membrane structure.

NF has been shown to increase the generation of activated oxygen species in several tissue microsomal preparations and to covalently bind to tissue macromolecules. Mason and Holtzman [2] have directly observed the one-electron reduction of NF to a nitroaromatic anion free radical in rat liver microsomes via EPR spectroscopy and proposed that this free radical reduces molecular oxygen to superoxide anion. Sasame and Boyd [3] observed the generation of elevated levels of both superoxide anion and hydrogen peroxide in rat lung microsomes incubated aerobically in the presence of NF, whereas under anaerobic conditions NF was found to bind covalently to microsomal macromolecules [4].

Drugs which cause hemolysis of G-6-PD-deficient red cells *in vivo*, such as NF, fail to cause hemolysis in either normal or G-6-PD-deficient red cells *in vitro* [1]. When G-6-PD-deficient red cells are incubated with NF *in vitro*, reintroduction of the cells into the

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§ Abbreviations: NF, nitrofurantoin; GSH, reduced glutathione; GSSG, oxidized glutathione; G-6-PD, glucose-6-phosphate dehydrogenase; GAPD, glyceraldehyde-3-phosphate dehydrogenase; and PGK, 3-phosphoglyceric phosphokinase.

donor results in hemolysis, suggesting a requirement for the participation of the reticuloendothelial system [5]. The mechanism of NF-mediated hemolysis may, therefore, involve drug-induced membrane damage which is recognized by the reticuloendothelial system.

Recent work in our laboratory has shown that NF interacts with purified human oxyhemoglobin and oxyhemoglobin in red cell hemolysates to cause the concomitant formation of methemoglobin and superoxide anion [6, 7]. To investigate further whether such an effect may occur within the normal human red cell, we have undertaken a study of the ability of NF to stimulate hydrogen peroxide and methemoglobin formation in red cells and the consequent effects of such processes on cellular levels of GSH and ATP. We also investigated the effect of NF on the morphology of the normal human red cell *in vitro*. The results presented herein demonstrate that NF increased hydrogen peroxide and methemoglobin formation while decreasing cellular levels of GSH and ATP. Consistent with these metabolic alterations, NF also was found to increase the formation of red cell echinocytes.

MATERIALS AND METHODS

Chemicals. NF was a gift from Norwich-Eaton Pharmaceuticals. All other biochemical reagents were obtained from the Sigma Chemical Co., St. Louis, MO.

Blood collection and incubation. Blood was collected from healthy adult human volunteers, who had no recent history of drug use or blood abnormality. Citrate was used as the anticoagulant. The erythrocytes were separated from the plasma, washed three times in normal saline, and then resuspended in a buffer containing the following concentrations of solutes: 115 mM NaCl; 4 mM KCl; 1 mM $MgCl_2$; 0.3 mM Na_2SO_4; 20 mM monobasic sodium phosphate adjusted to pH 7.45 with NaOH; and 10 mM glucose. Incubations were performed at 37° with shaking and exposure to atmospheric oxygen. A hematocrit of 33% was used routinely in all experiments, except in those experiments measuring hydrogen peroxide where a 5% hematocrit was employed. During the longest incubations (21 hr), less than 1% of the red cells hemolyzed, and the extent of hemolysis was not affected by the presence or absence of NF. In the dose–response experiments, the maximum concentration of NF employed was 840 μM—the limit of its solubility in distilled water.

Determination of red cell hydrogen peroxide. Since hydrogen peroxide is quite reactive, a method was needed to quantitate the hydrogen peroxide generated as a function of time during an incubation period. The method of Cohen and Hochstein [8], which makes use of the established fact that 3-amino-1,2,4-triazole (3AT) is an irreversible inhibitor of catalase only in the presence of hydrogen peroxide, was used. Thus, incubation of red cells in the presence of an excess (50 mM) of 3-amino-1,2,4-triazole and the subsequent assaying of catalase activity provided a measure of the amount of hydrogen peroxide generated. Catalase activity in dilute hemolysates of the red cell suspensions was assayed as described by Cohen *et al.* [9]. To an aliquot of hemolysate diluted 1:1000 at 0° was added a solution of 6 mM hydrogen peroxide. The reaction was carried out for 3 min and then terminated with 6 M sulfuric acid. An excess of 0.01 N potassium permanganate was added to the solution, and the absorbance at 480 nm was measured. The amount of hydrogen peroxide remaining, and thus the catalase activity, was determined from a standard curve.

Determination of red cell methemoglobin. The method of Hegesh *et al.* [10] which quantitates the amount of methemoglobin via conversion of methemoglobin to cyanomethemoglobin, and subsequent measurement of the absorbance at 630 nm, was used, and methemoglobin was reported as the percentage of total hemoglobin in the sample.

Determination of red cell GSH. The method of Beutler *et al.* [11] was used in the quantitation of red cell GSH levels. An aliquot of the red cell suspension was deproteinized with a saturated solution of sodium chloride containing 1.7% metaphosphoric acid and 6 mM EDTA. GSH in the supernatant fraction reacts with 5,5-dithiobis-(2-nitrobenzoic acid) to yield an adduct with an absorbance at 412 nm. An extinction coefficient of 13,600 $M^{-1} cm^{-1}$ was used for quantitation of the adduct concentration at 412 nm.

Determination of red cell ATP. The procedure used was that described by Adams [12]. An aliquot of the red cell suspension was deproteinized with an equal volume of 12% trichloroacetic acid. Buffered 3-phosphoglyceric acid and NADH were added to the supernatant fraction. The absorbance at 340 nm was measured, a solution containing rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (GAPD) and yeast 3-phosphoglyceric phosphokinase (PGK) was added, and the absorbance measured again until a constant value was obtained. ATP in the supernatant fraction reacts with the 3-phosphoglycerate in the presence of PGK to yield ADP and 1,3-diphosphoglycerate. NADH reacts with 1,3-diphosphoglycerate in the presence of GAPD to yield NAD and glyceraldehyde-3-phosphate. Thus, the number of moles of NADH oxidized, determined from the decrease in absorbance at 340 nm, is equal to the number of moles of ATP in the sample.

Electron microscopy. Three volumes of 3% phosphate-buffered glutaraldehyde (pH 7.4) were added to the erythrocyte suspensions. After several inversions, the suspension was centrifuged and the cell pellet was resuspended in glutaraldehyde. After post-fixation with osmium tetroxide and dehydration through increasing concentrations of ethanol, the cells were critical-point dried, coated with gold-palladium [13], and examined with a Philips-500 scanning electron microscope.

The electron micrographs were analyzed for the incidence of echinocytes. A cell was graded I (normal biconcave appearance), II (any deviation from biconcavity) or III (echinocyte), and the percentage of each cell class was determined in a sample of several hundred cells from a particular incubation. The identity of the samples was unknown to the electron microscopist at the time of the examination and photography of the samples as well as to the individual grading the cells.

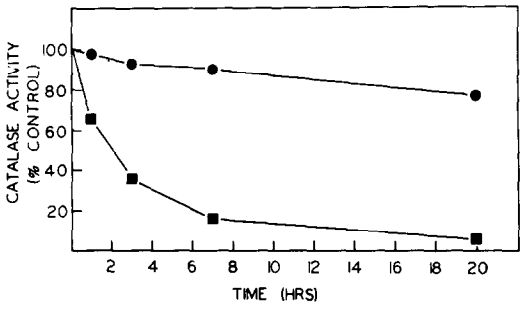

Fig. 1. Time-dependent increase in red cell hydrogen peroxide caused by NF. Red cells were incubated in the presence (■) and absence (●) of 840 μM NF, and catalase activity was assayed at various time points. Incubations also contained 50 mM 3-amino-1,2,4-triazole. Catalase activity is expressed as the percentage of activity in cells incubated in the absence of NF or 3-amino-1,2,4-triazole. Decreases in catalase activity are proportional to hydrogen peroxide generation. Each point represents the mean of four duplicate determinations performed on blood from four different donors.

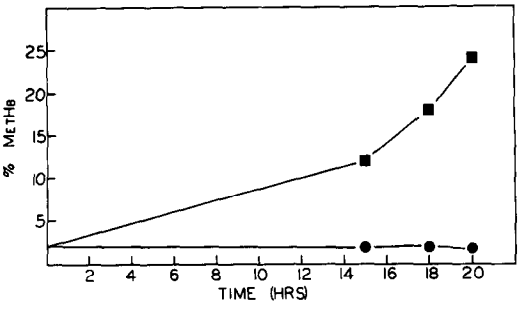

Fig. 2. Time-dependent increase in red cell methemoglobin caused by NF. Red cells were incubated in the presence (■) and absence (●) of 840 μM NF and assayed for methemoglobin at various time points. Each point represents the mean of duplicate determinations performed on blood samples from two different donors.

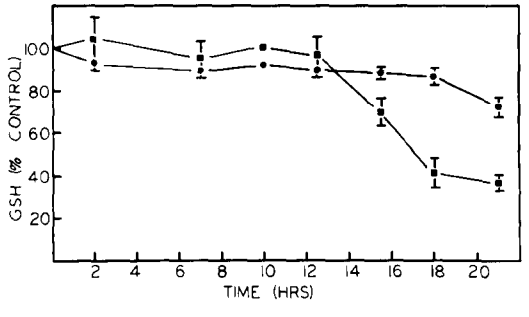

Fig. 3. Time-dependent depletion of red cell GSH by NF. Red cells were incubated in the presence (■) and absence (●) of 840 μM NF and assayed for GSH at various time points. Each point represents the mean ± S.E.M. for three to twelve experiments. The data are expressed as the percentage of the GSH level at zero time. The average initial GSH value was determined to be 0.488 ± 0.083 mole GSH/mole hemoglobin (mean ± S.D.).

RESULTS

Effects of NF on red cell hydrogen peroxide levels. Figure 1 shows the time-dependent decrease in catalase activity in red cells incubated with 3AT in the presence and absence of NF. Hydrogen peroxide was generated in the control blood as evidence by a ~20% decline in catalase activity during a 20-hr incubation. In contrast, red cells incubated under identical conditions but in the presence of NF lost over 95% of their catalase activity during the same period, indicating a substantially greater rate of hydrogen peroxide production.

Effects of NF on red cell methemoglobin levels. Figure 2 shows the effects of NF on cellular methemoglobin levels as a function of time. In the control blood, the methemoglobin concentration remained less than 2% throughout the duration of the incubation (20 hr). NF, however, caused a significant increase in the amount of methemoglobin, and more than 20% of the cell's hemoglobin had been oxidized to methemoglobin by 20 hr.

Effects of NF on red cell GSH levels. Figure 3 shows the effect of NF on red cell GSH levels as a function of time during a 21-hr incubation. The amount of GSH in the control cells remained fairly constant for about 18 hr and then fell slightly during the last 3 hr of the incubation. In the drug-treated blood, there was a reproducible, although statistically insignificant, increase in GSH levels as compared to control for about 12 hr. This was followed by a precipitous drop in the GSH levels such that by 21 hr the drug-treated blood had less than half the GSH levels as compared to the controls. Figure 4 shows the log dose–response relationship of the effect of NF on GSH depletion in red cells incubated for 21 hr. In this experiment, the EC_{50} was approximately 20 μM, a concentration of NF comparable to that achieved in blood after conventional doses of the drug [14].

Effects of NF on red cell ATP levels. Figure 5 shows the effects of NF on red cell ATP levels as a function of time during a 21-hr incubation. In the control blood, the ATP levels followed a pattern similar to that observed for GSH levels, remaining fairly constant for approximately 18 hr and decreas-

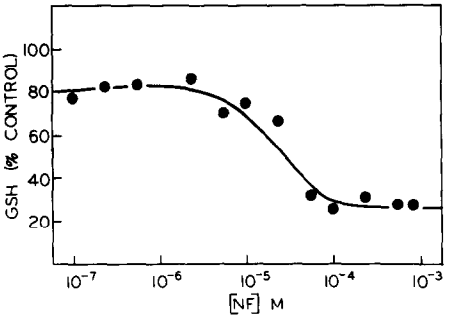

Fig. 4. Dose-dependent depletion of red cell GSH by NF. Red cells were incubated for 21 hr in the presence of various concentrations of NF and assayed for GSH. Each point represents the mean of duplicate determinations on blood samples obtained from the same donor as employed for experiments shown in Fig. 3.

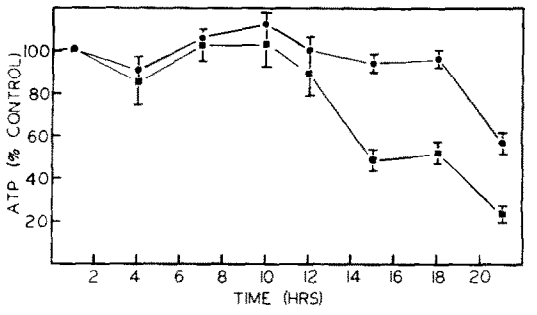

Fig. 5. Time-dependent depletion of red cell ATP by NF. Red cells were incubated in the presence (●) and absence (■) of 840 μM NF and assayed for ATP at various time points. Each point represents the mean ± S.E.M. for six to fifteen experiments. The data are expressed as the percentage of the ATP level at zero time. The average initial ATP value was determined to be 0.259 ± 0.029 mole ATP/mole hemoglobin (mean ± S.D.).

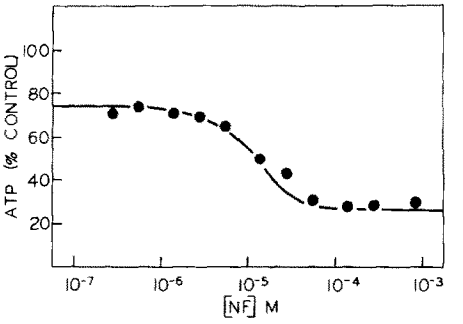

Fig. 6. Dose-dependent depletion of red cell ATP by NF. Red cells were incubated for 21 hr in the presence of various concentrations of NF and assayed for ATP. Each point represents the mean of duplicate determinations on blood samples from an individual donor.

ing by about 25% at 21 hr. In contrast, the cells incubated in the presence of NF were markedly depleted of ATP with significant decreases in ATP levels first manifested at approximately 10 hr; by 21 hr the ATP levels in the NF-treated blood were less than one-half those in the control cells. Figure 6 shows the log dose–response relationship of the effect of NF on ATP levels in erythrocytes incubated in the presence of drug for 21 hr. In this experiment, the EC_{50} was approximately 10 μM, a concentration of NF comparable to that achieved in blood after conventional doses of the drug [14]. These red cells were obtained from the same donor as those employed in the GSH dose–response studies (Fig. 4). Moreover, the EC_{50} values for ATP and GSH depletion were generally comparable for blood obtained from an individual donor. In experiments on blood samples obtained from different donors, a range of 10 to 100 μM was obtained for the EC_{50}, with the majority of the values occurring nearer 10 μM.

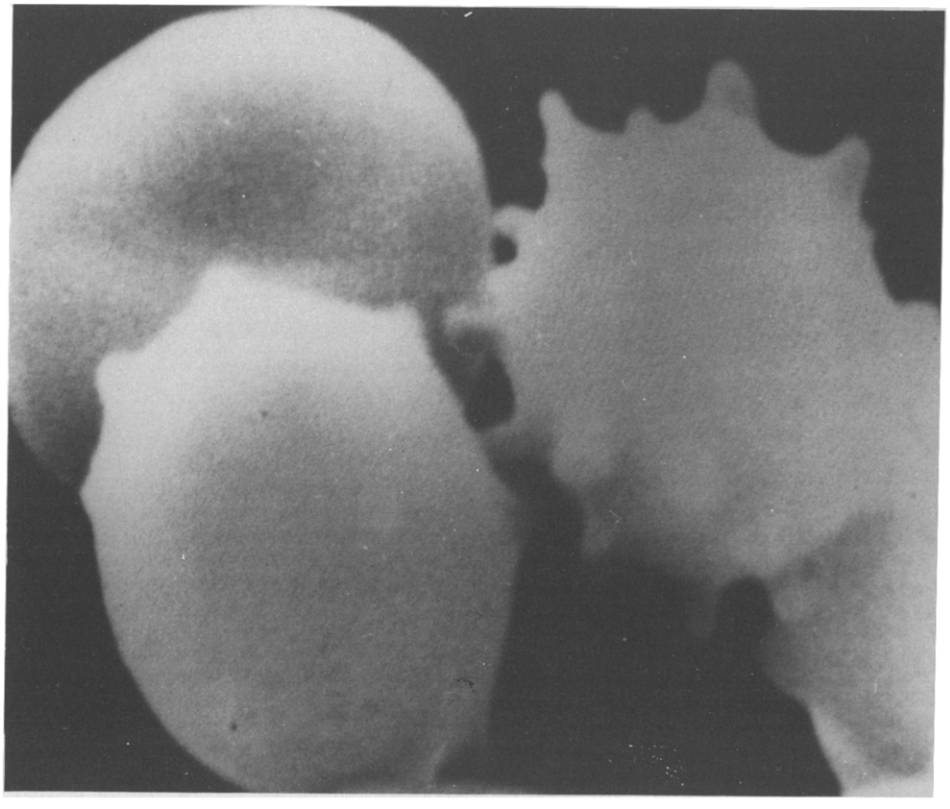

Fig. 7. Scanning electron micrograph of examples of the three classes of red cells. I, normal biconcave disc, upper left; II, deviation from normal biconcave disc, lower left; III, echinocyte, upper right. Magnification: × 10,000.

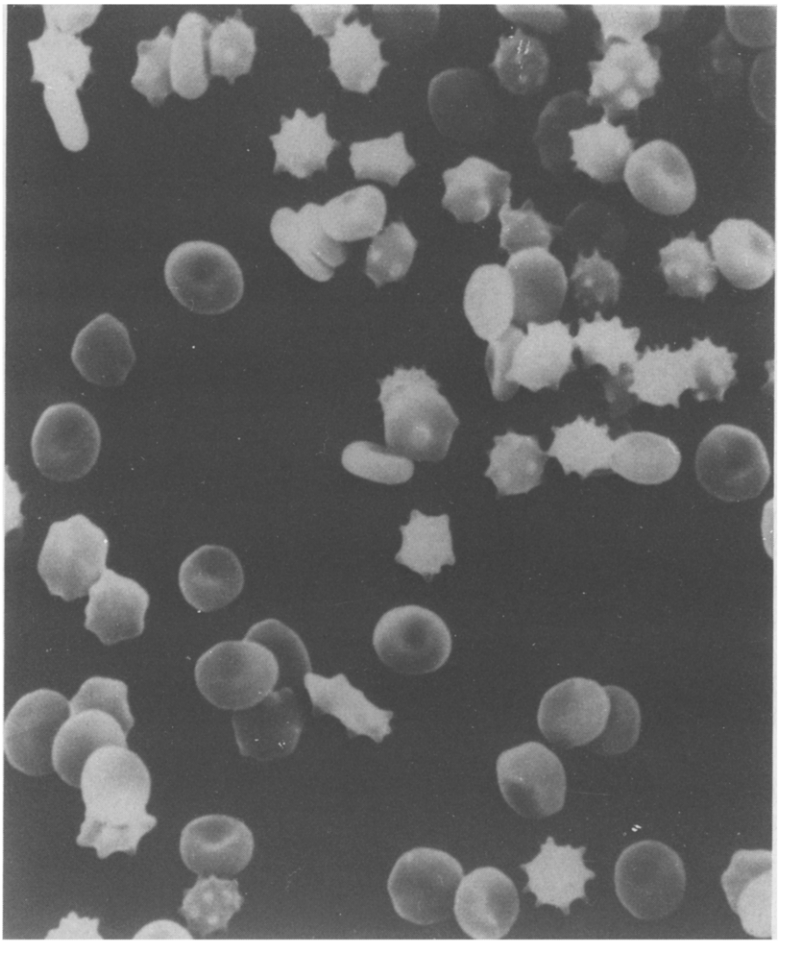

Fig. 8. Scanning electron micrograph of control red cells. Red cells were incubated for 21 hr in the absence of drug. Magnification: × 1250.

Effects of NF on red cell morphology. Figure 7 is a scanning electron micrograph of the three classes of cells. Figure 8 shows a scanning electron micrograph of cells incubated aerobically in the absence of drug for 21 hr, while Fig. 9 shows cells that had been incubated aerobically with NF for the same length of time. It is evident that most of the cells in Fig. 9 are abnormal in appearance, and biconcave discs are few in this field. Table 1 lists the percentages of each cell class in the various incubations. Although the effect of incubating the cells in the absence of NF was to cause the formation of a significant number of echinocytes, the presence of NF in the incubation resulted in a substantial increase in the percentage of echinocytes. Furthermore, an anaerobic environment significantly decreased echinocyte formation in the absence of drug but in the presence of NF a marked increase in abnormal cells was observed (Table 1). This is consistent with metabolic observations in that an anaerobic environment protected against ATP and GSH depletion in the absence of NF, whereas the presence of drug decreased both ATP and GSH levels to values comparable to those observed in an aerobic environment [7].

DISCUSSION

These data show that NF produced alterations in cellular metabolism in normal human red cells *in vitro*. NF caused the erythrocytic levels of hydrogen peroxide and methemoglobin to increase while causing a concomitant decrease in cellular concentrations of GSH and ATP. These effects may be the basis for the hemolysis manifested in persons having red cells deficient in certain enzymes which results in a predisposition toward lower levels, or rates of synthesis, of GSH.

When red cells were incubated with NF, the time course of the NF-induced metabolic changes was such that hydrogen peroxide generation occurred within a relatively short period of time and progressed rapidly, whereas significant alterations in cellular GSH, ATP and methemoglobin levels did not occur until 12–18 hr. When NF is prescribed for therapeutic use in humans, it is typically administered every 6 hr and, under these conditions, red cells *in vivo* are exposed to intermittent concentration pulses of the drug. In a G-6-PD-deficient person, several days are required prior to clinical

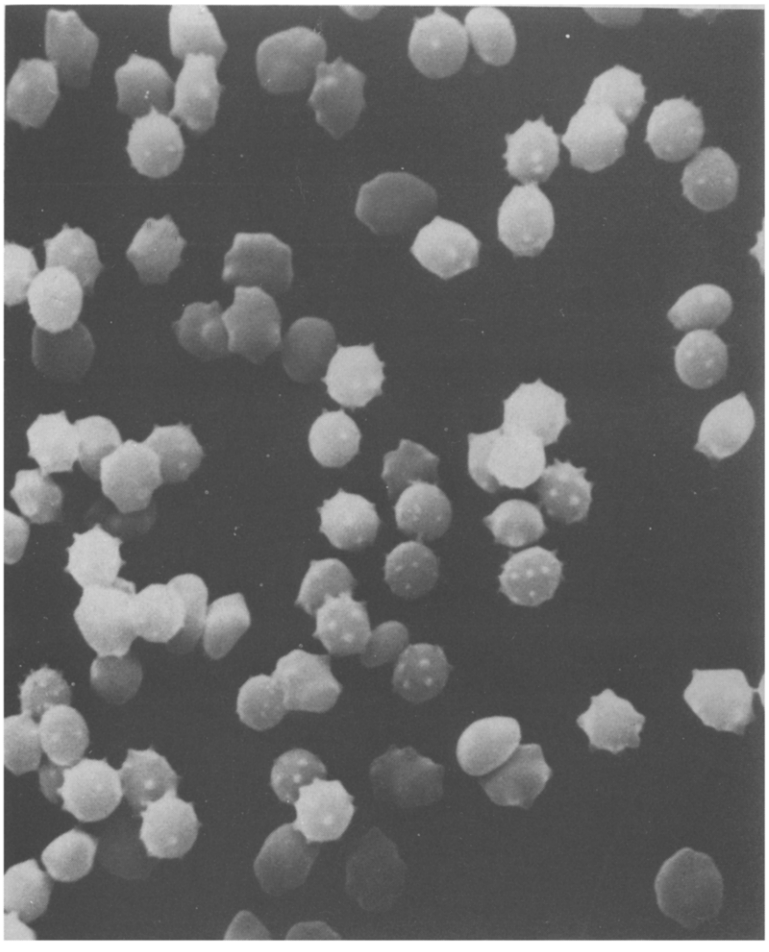

Fig 9. Scanning electron micrograph of NF-incubated red cells. Red cells were incubated for 21 hr in the presence of 840 μM NF. Magnification: $\times$ 1250.

Table 1. Effects of NF on the percentage of various cell classes*

Conditions	% Cell classes I	II	III
Aerobic			
T = 0 Control	91	6	3
T = 21 hr, control	51†	25†	24†
T = 21 hr, NF-treated	13†	26†	61†
Anaerobic			
T = 21 hr, Control CO atmosphere	90	9	1
T = 21 hr, NF-treated, CO atmosphere	26†	28†	46†

* Incubations were performed at 37° using a 33% hematocrit and 840 μM NF. Anaerobic experiments were performed using sealed vials. The red cell suspensions which contained the drug were gently bubbled with carbon monoxide for several minutes prior to being sealed. A slight positive pressure of carbon monoxide was maintained in the vial throughout the duration of the experiment.

† Significantly different from T = 0 control and the T = 21 hr control with carbon monoxide atmosphere and also significantly different from each other using the Mann–Whitney rank test ($P < 0.0005$).

manifestations of hemolytic anemia caused by NF [15]. Thus, although the molecular event(s) ultimately leading to NF-mediated cellular toxicity appears to begin immediately, a significant delay period is required until the toxicity is expressed at the cellular level. Presumably this delay is related to that time during which the cell loses its ability to protect itself against oxidative stress, perhaps by depletion of protective cofactors such as GSH. Furthermore, the EC_{50} values for GSH and ATP depletion obtained in these studies are comparable to the blood concentrations achieved with the usual therapeutic doses of the drug which are capable of causing hemolytic anemia in G-6-PD-deficient persons.

Previous work by other investigators has demonstrated that, in microsomes, the primary metabolic pathway for NF is reduction of the nitro group [2, 4]. The nitro anion free radical thus generated, or a subsequent reduction product, has been proposed to bind covalently to cellular macromolecules in the absence of molecular oxygen [4], while under aerobic conditions, the free radical may reduce oxygen to superoxide anion [2]. Superoxide anion may dismutate for form hydrogen peroxide, and the combined presence of superoxide and hydrogen peroxide may lead to an iron-catalyzed Haber–Weiss reaction resulting in the formation of hydroxyl radicals or other potent oxidants capable of peroxidizing membrane lipids, a process which in red cells may damage the membrane and result in hemolysis. Furthermore, the covalent binding of a reactive metabolite of NF to the red cell membrane could conceivably cause structural alterations which subsequently result in hemolysis. Hochstein and Rice-Evans [16] have provided evidence that lipid peroxidation of endogenous membrane lipids in erythrocytes may be a contributing factor in decreasing cellular deformability (i.e. increasing rigidity) which may contribute to red cell aging and destruction by the recticuloendothelial system.

Although the red cell does not contain microsomal enzymes, it does contain enzymes capable of bioactivating NF to reactive intermediates. The red cell contains several reductase enzymes such as NADH- and NADPH-dependent methemoglobin reductases, and glutathione reductase, whose primary purpose appears to be the reduction of methemoglobin and oxidized glutathione respectively. One or more of these enzymes could reduce NF to the free radical species resulting in covalent binding and/or superoxide and hydrogen peroxide generation. Our previous work has shown that the direct interaction of NF and oxyhemoglobin results in the generation of superoxide anion with formation of methemoglobin [6, 7]. We have also reported that covalent binding of NF to red cells occurs under both anaerobic and aerobic conditions although it does not appear to be a significant process [7]. Nevertheless, it is likely that both superoxide generation and covalent binding may occur with NF in the intact red cell by one or more mechanisms and such effects would be responsible for the metabolic and morphologic alterations we have observed.

We have shown that NF increases the levels of methemoglobin and hydrogen peroxide in the red cell. These effects may occur via one or both of two mechanisms. First, if NF were reduced to a free radical in the presence of oxygen, superoxide is likely to be generated via transfer of the electron to molecular oxygen. Superoxide anion may enzymatically dismutate to hydrogen peroxide or it may interact with oxyhemoglobin to yield methemoglobin and hydrogen peroxide [17]. Second, the direct interaction of NF and oxyhemoglobin yields methemoglobin, superoxide, and hydrogen peroxide [6]. In another study, we present evidence assigning a greater level of importance to the latter mechanism [7]. For example, ethyl isocyanide, a ligand of ferrohemeproteins which binds hemoglobin with high affinity and prevents oxygen binding, and 2′-AMP, an inhibitor of NADPH-dependent reductase enzymes, both protect against NF-mediated GSH depletion in the red cell. When red cells are incubated with 840 μM NF in the absence and presence of ethyl isocyanide (27 mM) or 2′-AMP (10 mM), GSH levels increase significantly (i.e. 2- to 3-fold when expressed as percent of control) [7]. Since GSH levels fall in response to activated oxygen formation, the mechanism involving NF-mediated superoxide release from oxyhemoglobin appears to be responsible for a significant amount of the superoxide generated by NF within the red cell. Thus, NF produces a significant increase in red cell hydrogen peroxide and methemoglobin levels and the importance associated with such an increase in generation of activated oxygen species (e.g. hydrogen peroxide) lies in the ultimate effect of these species in depleting red cell GSH levels and producing red cell toxicity.

Three mechanisms may be operative in the NF-mediated depletion of cellular GSH. These include (1) an increase in activated oxygen species formation, (2) inhibition of glutathione reductase, and (3) the covalent binding of a reactive NF intermediate to GSH. First, the generation of superoxide and hydrogen peroxide should result in a decrease in the levels of GSH. GSH may react with superoxide to yield oxygen and the GS·radical. Two GS·radicals may then react forming oxidized glutathione, GSSG. Furthermore, the reduction or dismutation of superoxide yields hydrogen peroxide. An important metabolic pathway for hydrogen peroxide detoxification is its reduction to water via glutathione peroxidase which utilizes GSH as the source of reducing equivalents. The effects of NF on GSH levels can thus be explained on the basis of the action of NF in causing activated oxygen formation. Second, NF is also a known inhibitor of glutathione reductase [18], and hence the NF-mediated increase in generation of activated oxygen species such as hydrogen peroxide may be exacerbated by concomitant inhibition of glutathione reductase. Third, GSH is a known scavenger of free radical drug metabolites. Thus, the formation of the NF free radical may also result in depletion of GSH via covalent binding of the NF free radical to GSH.

The depletion of ATP may result from effects related to an increased rate of GSH oxidation or from damage to hemoglobin, red cell proteins, or the erythrocyte membrane. ATP is generated in the red cell solely via the Embden-Meyerhof pathway which uses glucose-6-phosphate as its energy

substrate. Normally, about 85–90% of the glucose-6-phosphate in the red cell enters this pathway. The remaining glucose-6-phosphate enters the hexose monophosphate shunt which results in the generation of NADPH. Whenever GSH oxidation increases, NADPH utilization also increases, and a greater percentage of glucose-6-phosphate enters the hexose monophosphate shunt. Thus, there is less glucose-6-phosphate available for ATP generation in the Embden–Meyerhof pathway, and the ATP levels are likely to fall. Furthermore, one purpose of ATP in the red cell is to maintain the concentration gradients of sodium and potassium ions across the cell membrane via Na^+, K^+-ATPase. If NF were to damage the plasma membrane in such a way as to result in an increase in membrane permeability to ions, an increased rate of ATP utilization may be required to maintain the gradient, and the ATP levels within the cell may also decrease.

In addition to the proposed mechanism(s) which may be operative, the drug-mediated depletion of ATP and GSH is likely to compromise the survival of the erythrocyte. Since ATP is of utmost importance in maintaining the ionic gradient across the plasma membrane, its depletion would result in an increase in the intracellular concentration of sodium ion and a decrease in the intracellular concentration of potassium ion. The decrease in the intracellular potassium ion concentration could subsequently diminish the activities of various important enzymes, such as pyruvate kinase. Furthermore, a substantial decrease in intracellular ATP could also impair the ability of the cell to utilize glucose for ATP generation via the Embden–Meyerhof pathway and NADPH generation via the hexose monophosphate shunt because the *first* step in the metabolism of glucose, catalyzed by hexokinase, *requires* ATP. The drop in intracellular GSH levels would, as previously discussed, impair the ability of the cell to detoxify activated oxygen species which could then damage the cell membrane or oxidize oxyhemoglobin to methemoglobin.

Normal human whole blood, incubated at 37° for 24 h [19] or at 4° for 2–4 weeks [20], has a large number of echinocytes. This transformation from discocyte to echinocyte is likely to result from depletion of intracellular ATP [19–21]. Thus, our data, which show that NF increased the number of echinocytes *in vitro*, coupled with our observation that NF caused the cellular depletion of ATP, suggest that drug-mediated depletion of ATP may be responsible for the conversion of the cells to echinocytes.

The occurrence of echinocytes *in vivo* is rare, if at all [22, 23]. NF-damaged enzyme-deficient erythrocytes, however, may be hemolyzed via the action of the reticuloendothelial system [5]. The recognition of such cells is likely to depend on some membrane alteration; thus, the membrane changes which have been observed after incubation of normal human red cells with NF may be related to what occurs to G-6-PD-deficient cells in a person administered NF.

Persons having deficiencies in enzymes important in the protection of the cell against oxidative damage (i.e. GSH reduction or synthesis) due to free radical species are likely to suffer hemolytic episodes when administered NF. Thus, the toxic effect of NF is probably related to the formation of reactive intermediates dependent upon GSH for detoxification. In support of such a hypothesis, we have shown that NF caused activated oxygen formation and GSH depletion in normal human red cells as well as the expected sequelae of the formation of methemoglobin and the depletion of ATP.

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