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METHYLENE BLUE-MEDIATED HEXOSE MONOPHOSPHATE SHUNT STIMULATION IN HUMAN RED BLOOD CELLS IN VITRO: INDEPENDENCE FROM INTRACELLULAR OXIDATIVE INJURY*

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Abstract—1. The red blood cell hexose monophosphate shunt (HMS) and proteolytic responses to several concentrations of Methylene Blue or sodium nitrite were measured.

2. The results suggested two distinct mechanisms for activation of the HMS: (1) nitrite treatment increased HMS activity in response to oxidative challenge to red cell protein; (2) Methylene Blue treatment activated HMS without injurious oxidative challenge. Nitrite-treated cells actively degraded protein, whereas Methylene Blue-treated red cells did not activate proteolytic systems that degrade oxidized red cell protein.

3. These observations are relevant to proposed *in vitro* systems for evaluation of drug hemolytic toxicity potential on the basis of HMS stimulation capacity.

INTRODUCTION

Persons suffering from an inherent deficiency of red cell glucose-6-phosphate dehydrogenase (G6PD) are often susceptible to acute intravascular hemolysis following treatment with certain drugs (Carson and Frischer, 1966; Kirkman, 1968; Beutler, 1969). The deficiency diminishes the ability of the erythrocyte to accelerate the hexose monophosphate shunt (HMS) and, therefore, to sustain adequate levels of reduced nicotinamide adenine dinucleotide phosphate (NADPH) under oxidizing conditions (Gaetani *et al.*, 1974) (see Fig. 1). Since NADPH is required for reduction of glutathione, and reduced glutathione serves to detoxify peroxide and to protect protein sulfhydryl groups from oxidation, G6PD-deficient erythrocytes have a reduced ability to detoxify an oxidative challenge (Fraser and Vessell, 1968; Kirkman *et al.*, 1975). Upon this basis many drug-induced hemolyses have come to be regarded as a consequence of intracellular oxidative attack (Keller, 1971; Gordon-Smith, 1980; Carson *et al.*, 1981).

It has been proposed that the capability of a drug to stimulate the HMS in normal red cells would reflect a potential for hemolytic toxicity in G6PD-deficient erythrocytes (Welt *et al.*, 1971; Gaetani *et al.*, 1976; Pescarmona *et al.*, 1982). Since it is not the HMS *per se* that reverses oxidative damage, one must presume that the elevation of HMS activity observed under drug treatment is directly coupled to oxidative challenge (i.e. to glutathione redox flux, see Fig. 1) to relate such predictions to oxidative toxicity. If mechanisms are operative through which the HMS may be stimulated in the absence of oxidative challenge,

HMS stimulation would not necessarily be indicative of drug toxicity.

Roth *et al.* (1975) and Carson *et al.* (1981), have recognized at least two mechanisms through which the HMS is activated in erythrocytes, one of which appears to function independently of glutathione redox flux. Some drugs activate the HMS in the presence of a specific inhibitor of glutathione reductase, whereas other drugs fail to do so under the same conditions. Carson *et al.* (1981) designated these groups of compounds as "Methylene Blue-like" or "ascorbate-like" respectively. The former group contained those compounds that maintained or increased HMS activity in the presence of the inhibitor, and the latter group contained compounds that did not maintain elevated HMS activity under glutathione reductase inhibition.

The results presented in the present report provide additional support for the proposed existence of two distinct HMS stimulation mechanisms, one of which is independent of glutathione redox flux. The data on proteolysis were particularly useful to this interpretation because the proteolytic system is activated by oxidation of protein (Goldberg and Boches, 1982) and, therefore, is a measure of real oxidative damage to red cell protein.

METHODS

Proteolysis measurement

Buffers. Physiologically balanced salts solution (PBSS) and phosphate buffered salts solution (PBS) were prepared as described previously (Baird *et al.*, 1984).

Reagents. Stock solutions of HMS stimulants; 250 mM sodium nitrite (reagent grade, Aldrich Chemical Co., Milwaukee, Wisconsin, U.S.A.) and 250 μ M Methylene Blue (3,7-bis(dimethylamino) pherothiazin-5-ium chloride, pharmaceutical grade, Mallinkrodt Chemical Works, St Louis,

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Fig. 1. The hexose monophosphate shunt and glutathione redox flux. Sequence A represents glutathione mediated enzymatic reduction of hydrogen peroxide to water and sequence B represents the spontaneous reduction of oxidized protein sulfhydryl groups, the two means by which glutathione protects red cells against oxidative damage. GSH, reduced glutathione; GS-SG, oxidized glutathione; P-SH, reduced protein sulfhydryl; P-S-S-P, oxidized protein sulfhydryl; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NADP⁺, oxidized NADPH. (1) hexokinase; (2) glucose-6-phosphate dehydrogenase; (3) glutathione reductase; (4) glutathione peroxidase; (5) 6-phosphogluconolactonase or spontaneous hydrolysis; (6) 6-phosphogluconate dehydrogenase.

Missouri, U.S.A.) were prepared as previously described (Baird *et al.*, 1984).

Reagents for tyrosine assay, 1-nitroso-2-naphthol (reagent grade, Aldrich Chemical Co.) and nitric acid (Aldrich Chemical Co.), were also prepared as described elsewhere (Waalkes and Udenfriend, 1957).

Red blood cells. Twenty ml whole blood was drawn by venipuncture from a 24-year-old Caucasian male and immediately washed and albuminized as reported previously (Baird *et al.*, 1984). Cycloheximide (Aldrich Chemical Co.) was added to the red cell suspension (0°C) to 0.5 mM final concentration to prevent incorporation of liberated tyrosine into reticulocyte protein (Goldberg and Boches, 1982). Concentration of RBC suspensions added to reaction mixtures were approximately those found in whole blood.

Incubation. Two ml PBSS containing 10 mM D-glucose (reagent grade, Sigma Chemical Co., St Louis, Missouri, U.S.A.) served as the incubation medium. One ml of HMS stimulant solution was added to the medium prior to addition of 1 ml erythrocyte suspension. The incubation vessels were 10 ml polystyrene screw-cap test tubes placed horizontally at 37°C and ambient atmosphere for 30 min.

Incubation was terminated by addition of 4 ml cold (4°C) 30% trichloroacetic acid. Acid treated samples were stored at 4°C overnight.

Tyrosine assay. Acid-treated samples were centrifuged at 1600g for 10 min. Two ml of supernatant were placed in a 50 ml polypropylene centrifuge tube (Falcon 2070 Tube, Beckton, Dickinson and Co., Oxnard, California, U.S.A.) followed by addition of 1 ml nitroso-naphthol reagent and finally 1 ml nitric acid solution. The reaction mixture was placed in a 55°C water bath for 30 min. Immediately following incubation, solutions were cooled to room temperature and 10 ml ethylene dichloride (spectrophotometric grade, Aldrich Chemical Co.) were added. Mixtures were shaken vigorously for about 10 sec and centrifuged at 1600g for 10 min. Two ml of the aqueous layer were removed for spectrophotofluorometric measurement of the tyrosine-naphthol complex in an Aminco Bowman spectrophotofluorometer. Excitation wavelength was set at 465 nm and

emission intensity was measured at 565 nm as described previously (Waalkes and Udenfriend, 1957).

HMS activity measure

HMS activity was measured, with correction for glucose carbon recycling through the shunt, as described previously (Baird *et al.*, 1984). To estimate the possible influence of 0.5 mM cycloheximide upon HMS stimulation induced by Methylene Blue or sodium nitrite, cycloheximide-treated red cells were treated separately with these compounds as described above for measurement of proteolysis and HMS activities.

Statistical analysis

Statistical analyses were conducted as previously described (Baird *et al.*, 1984).

RESULTS

Proteolysis measurements

Release of tyrosine from red cells in the presence of increasing concentrations of Methylene Blue or sodium nitrite is shown in Fig. 2. Tyrosine concentrations of non-treated erythrocyte suspensions averaged 3 nmol/ml. This was taken to represent tyrosine from sources other than oxidant-induced proteolysis (see "Discussion") and was set to equal a baseline proteolytic activity of zero.

Methylene Blue induced no proteolytic activity under any test conditions. Nitrite-treated cells released tyrosine at maximum rates of 25 and 24 nmol·ml RBC⁻¹·hr⁻¹ with 20 and 40 mM NaNO₂, respectively. Proteolytic activity was observed at 5, but not 2.5 mM NaNO₂. Treatment groups were run in duplicate and on two separate occasions. The results obtained in these experiments were in good agreement.

Fig. 2. Proteolytic activity in red cell suspensions treated with sodium nitrite (●) or with Methylene Blue (○) as determined by monitoring the proteolytic release of tyrosine from erythrocyte protein by spectrophotofluorometric assay.

HMS activity measurements

Stimulation of HMS activity by sodium nitrite is shown in Fig. 3. Increases in activity between 0, 0.625, 1.88 and 2.50 mM NaNO_2 were significant ($p < 0.001$). HMS activity became maximal at approx. 65 activity units (nmol CO_2 trapped · ml $\text{RBC}^{-1} \cdot \text{hr}^{-1}$) in red cells treated with between 10 and 20 mM NaNO_2 . The difference between these means was insignificant ($P > 0.1$). The 37% decrease in activity between 20 and 80 mM NaNO_2 was significant ($P < 0.001$). Appreciable hemolysis was not observed in any NaNO_2 -treated RBC suspension.

Stimulation of HMS activity by Methylene Blue is shown in Fig. 3. The rate of activity reached a maximum at approx. 120 activity units following treatment with between 10 and 80 μM Methylene

Fig. 3. Hexose monophosphate shunt (HMS) activity in red cell suspensions treated with sodium nitrite (mM (●)) or with Methylene Blue (μM (○)). The data points are the sum of activity measurements for both primary HMS activity and HMS activity due to glucose carbon recycling through the HMS in which means were obtained from replicate sets of 10 samples each. Brackets indicate the limits for the 95% confidence interval.

Blue, as evidenced by the insignificant ($P > 0.05$) differences among these means.

Based on calculation from maximal HMS activities with minimal oxidant concentration, 200 mol NaNO_2 are required to stimulate the HMS to release 1 mol CO_2 . Methylene Blue, however, induced the HMS to release 160 mol CO_2 per mol Methylene Blue. Thus, on a molar basis, Methylene Blue chloride is 3.2×10^4 -fold more potent an HMS stimulant than sodium nitrite.

These experiments were conducted on two separate occasions (data not given) and the activity means were not significantly different ($P > 0.05$) from those given herein. Differences between the HMS activity means of cycloheximide-treated and non-treated red cells were insignificant ($P > 0.05$) in the absence and presence of HMS activity-saturating concentrations of sodium nitrite or Methylene Blue (data not given).

DISCUSSION

Measurements of proteolytic and HMS activities have been used to characterize red blood cell responses to sodium nitrite and to Methylene Blue chloride. The responses to sodium nitrite were consistent with those expected from injurious oxidative attack. Methylene Blue treated red cell suspensions, however, did not respond similarly. The results indicate that HMS stimulation observed under Methylene Blue treatment occurs in the absence of a nitrite-like oxidative attack. This observation is relevant to proposed *in vitro* systems which would evaluate drug potential for hemolytic toxicity in G6PD-deficient persons based upon capacity of drug for HMS stimulation.

Oxidation of protein sulfhydryl groups can result in loss of functional protein conformation. Reduced glutathione (GSH) is a highly reactive peptide (γ -L-glutamyl-L-cysteinylglycine) which serves to guard against such events through two means: (1) direct reduction of oxidized protein sulfhydryls; (2) enzymatic detoxification of hydrogen peroxide which may otherwise oxidize protein sulfhydryls and generate oxidizing radicals (Beutler, 1980; Srivastava *et al.*, 1980). As an intracellular oxidative potential is increased, the GSH oxidation rate is accelerated and NADPH is consequently more rapidly oxidized to NADP^+ in catalysis of oxidized glutathione (GSSG) reduction (Kirkman *et al.*, 1980). When the oxidative potential is of such magnitude that the rate of production of GSSG exceeds that of GSH, the latter will become depleted and oxidation of protein may more readily occur (Srivastava *et al.*, 1980). These reactions are directly coupled to the HMS (see Fig. 1).

The recent report by Goldberg and Boches (1982) has revealed the existence of an ATP-dependent proteolytic system in mature red cells that is activated in the presence of abnormal protein side group moieties, such as those generated during oxidative attack. In the present study, activity of this proteolytic system has been measured to provide an estimate of oxidative damage to erythrocyte cytoplasm. In accordance with the processes described in the paragraph above, it may reasonably be presumed that activation of the proteolytic system would occur only

when intracellular GSH has been depleted. That is, in the presence of a high GSH/GSSG ratio, protein would be protected against oxidation and the proteolytic system would remain inactive. It is thus evident that a proteolytic response is indicative of failure of the glutathione redox system to prevent an injurious oxidative attack.

The nitrite-HMS activation plot (Fig. 3) and the nitrite-induced proteolytic responses (Fig. 2) are consistent with a generalized oxidative attack. The slow initial rate of HMS activation seen in Fig. 3 probably reflects a buffering effect provided by the very high ambient GSH/GSSG ratio (Beutler, 1980), i.e. the oxidation potential introduced by 2.5 mM or less NaNO_2 (Fig. 3) appears to have been effectively reduced without causing an appreciable accumulation of NADP^+ (i.e. HMS stimulation). On the other hand, 5 mM NaNO_2 (Fig. 3) generated peroxide (Cohen *et al.*, 1964) and oxidized protein at a rate which probably elicited GSH oxidation in sufficient quantity to increase demand for NADPH to a level which could not be immediately satisfied. The result would be a fall in the $\text{NADPH}/\text{NADP}^+$ ratio and subsequent HMS stimulation (Gaetani *et al.*, 1974; Kirkman *et al.*, 1980).

The nitrite-induced proteolysis responses are consistent with this interpretation. In Fig. 2 one may see that concentrations of nitrite below HMS activity saturation potential did not induce proteolysis and thus appear to have not exhausted the reduction potential of GSH. At nitrite concentrations of 10 mM and above (at and above HMS activity maximum, see Fig. 3) proteolytic activity was observed. This effect may be interpreted quantitatively as a response to oxidative attack; at maximal HMS activity levels the $\text{NADPH}/\text{NADP}^+$ ratio is very low (Kirkman *et al.*, 1980) so that effective GSH protection against oxidation may be lost and oxidative denaturation of erythrocyte protein would occur (Srivastava *et al.*, 1980), thus creating substrate for the ATP-dependent proteolytic system. Decreases observed in HMS activity between 20 and 80 mM NaNO_2 may be the result of oxidative/proteolytic decomposition of red cells.

Red cells treated with Methylene Blue did not respond as would be expected after exposure to an injurious oxidative potential. If the Methylene Blue-induced HMS stimulation was elicited for the purpose of protection against a potentially toxic oxidative challenge, 0.625 μM Methylene Blue would have to introduce an intracellular oxidizing potential equivalent to that introduced by 5 mM NaNO_2 because at both of these concentrations the HMS was elevated to approx. 30 activity units. This seems improbable because nitrite has an oxidation potential (1.0 volt) much greater than that of fully oxidized Methylene Blue (0.065 volt) (Long, 1961). Despite the more powerful oxidation potential nitrite possesses, Methylene Blue was remarkably more effective in inducing glucose catabolism through the HMS (3.2×10^4 -fold). The relatively potent effect of Methylene Blue on HMS activity must be due to its ability to accept electrons from NADPH via diaphorase II (Harvey and Kaneko, 1977). In this manner Methylene Blue may induce HMS stimulation through generation of NADP^+ (Gaetani *et al.*, 1974) and

subsequent activation of G6PD (Kirkman *et al.*, 1980). Metz *et al.* (1976) demonstrated that hemoglobin was an effective electron acceptor for the reduced Methylene Blue (Leukomethylene Blue) product of diaphorase II activity. Thus, minute quantities of Methylene Blue may be continuously reduced and oxidized to effect a strong HMS response (Harvey and Kaneko, 1977). This autooxidative process has been described as oxidatively stressful (Gaetani *et al.*, 1974; Metz *et al.*, 1976) because this reaction with hemoglobin may result in formation of superoxide which may gain electrons in its spontaneous reduction to water (Srivastava *et al.*, 1980). This process (superoxide formation) has been documented in the reaction of nitrite with oxyhemoglobin (Tomada *et al.*, 1981; Doyle *et al.*, 1982). In general, if electrons for superoxide reduction are obtained randomly from oxidatively labile groups in the erythrocyte, rather than GSH, an oxidative attack results (Srivastava *et al.*, 1980). Thus, if the redox flux of Methylene Blue generated toxic quantities of oxidizing radicals, oxidative denaturation of red cell protein would be expected. The proteolysis measurements under Methylene Blue treatment do no support this interpretation. The absence of proteolysis in Methylene Blue-treated red cells (Fig. 2) suggests that the dye (and the hypothesized oxidation potential held in intracellular oxygen) did not challenge oxidatively labile protein groups even at concentrations as much as 8-fold in excess of that required for HMS activity saturation. These observations demonstrate that Methylene Blue-induced stimulation of the HMS is not associated with a toxic accumulation of oxygen radicals.

The trivial explanation for the absence of proteolytic activity in Methylene Blue-treated red cells might be that the dye either inhibited the proteolytic system or chemically interfered with recovery/measurement of the tyrosine-naphthol complex used to quantify proteolytic activity. These possibilities were considered unlikely, however, because in experiments in which Methylene Blue (12.5 μM) was added with 20 mM NaNO_2 red cell suspensions, no diminution of proteolytic activity was observed (data not given).

Data of previous studies suggest that Methylene Blue-induced HMS stimulation may occur in the absence of toxic oxidative challenge. Red cells treated with Methylene Blue, at concentrations comparable to those employed in this study, accumulate rather than consume GSH (Bockris and Smith, 1962; Jacob and Jandl, 1966). Also, these concentrations of Methylene Blue do not generate detectable levels of H_2O_2 (Smith and Mahaffey, 1977; Harvey and Kaneko, 1976). The data of Carson *et al.* (1981) discussed in the introduction to this report are also consistent with this interpretation; i.e. Methylene Blue-induced elevation of HMS activity was independent of GSH redox flux. Since Methylene Blue activates the HMS without introduction of a toxic oxidation potential, this HMS stimulation does not appear to be related to defense against oxidative injury.

This study has demonstrated that there exists at least one mechanism through which the red cell HMS activity level may become elevated in the absence of

a toxic oxidizing potential. Thus, HMS stimulation capacity of a drug may not serve as a reliable indicator of "hemolytic toxicity", as has been suggested (Welt *et al.*, 1971; Gaetani *et al.*, 1976; Pescarmona *et al.*, 1982). It would appear that those compounds designated "Methylene Blue-like" by Carson *et al.* (1981) may elicit HMS activity elevation without toxic oxidation and, therefore, may be unsuitable for use in hemolytic toxicity screening procedures utilizing the HMS stimulation capacity criterion.

In this regard, whether or not certain hemolytic drugs behave like Methylene Blue or sodium nitrite is an important question. Primaquine, a widely used antimalarial with known hemolytic toxicity, is a case in point. Cohen and Hochstein (1964) reported that this compound generated H_2O_2 in human red cells incubated *in vitro*, which would suggest sodium nitrite-like oxidative behavior. However, Kelman *et al.* (1982), have recently demonstrated that although primaquine does generate H_2O_2 , primaquine-induced HMS stimulation is independent from H_2O_2 generation and glutathione redox flux. The authors pointed out that primaquine appears to stimulate the HMS in much the same manner as Methylene Blue. Similarly, we have reported that two putative metabolites of primaquine (hydroxylated) show Methylene Blue-like oxidative activity in G6PD-normal and deficient red blood cells *in vitro*; potent HMS stimulation without causing proteolysis, and an ability to oxidize NADPH directly and via diaphorase II (Baird *et al.*, 1983). These findings suggest that evaluation of 8-aminoquinoline antimalarial drugs for hemolytic toxicity based on capacity for HMS stimulation *in vitro* may be unsatisfactory. Specifically, primaquine-induced HMS stimulation may not be correlated to a realized oxidative challenge to red cell protein.

SUMMARY

Data are presented which demonstrate that elevation of HMS activity by red blood cells may not be correlated to erythrotoxic oxidative processes *a priori*. *In vitro* measurements of the activity of a recently described proteolytic system, which rapidly degrades oxidized proteins in mature red blood cells, were employed to determine the extent of oxidative injury incurred during exposure to two HMS stimulants, sodium nitrite and Methylene Blue chloride. HMS activity was measured by quantitation of $^{14}CO_2$ from glucose radioisotopes added to red cell suspensions. The levels of proteolytic and HMS activities elevated by sodium nitrite appeared closely related; low levels of HMS activity (<10-fold elevation) were not associated with detectable proteolytic activity, but with increasing HMS activity (>15-fold elevation) proteolysis was evident. Even though Methylene Blue elevated HMS activity by as much as 45-fold, concentrations of this stimulant in as much as 8-fold excess of that required to induce this level of HMS activity (higher concentrations were not examined) did not cause proteolysis. It is thus evident that elevation of HMS activity is not necessarily related to toxic intracellular oxidative stress. Thus, proposed systems which would evaluate the oxidative toxicity of certain compounds on the basis of their

effect on the red blood cell HMS *in vitro* may be inappropriate. Primaquine, a hemolytically toxic antimalarial drug, has been examined in such systems and the fact that it is capable of HMS stimulation has been cited as indicative of its oxidative toxicity. Recent reports discussed herein suggest that the primaquine effect on the HMS is essentially Methylene Blue-like, and therefore estimations of its toxicity made on this criterion would appear invalid.

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