THE EFFECTS OF DRUGS ON ERYTHROCYTES *in vitro*: HEINZ BODY FORMATION, GLUTATHIONE PEROXIDASE INHIBITION AND CHANGES IN MECHANICAL FRAGILITY

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1 The relationship between mechanical fragility, glutathione peroxidase inhibition and Heinz body formation, in erythrocytes exposed to oxidant drugs *in vitro*, has been investigated. All drugs tested caused Heinz body formation, and with the exception of acetyl salicylic acid and salicylic acid, also caused increased erythrocyte mechanical fragility.

2 There was a direct relationship between mechanical fragility and drug concentration. Mechanical fragility increased in parallel with Heinz body formation, with primaquine, gentisic acid, ascorbic acid and potassium chlorate. In contrast Heinz body formation occurred at drug concentrations which did not cause a marked increase in mechanical fragility in the case of menadione, acetyl phenylhydrazine and phenylhydrazine.

3 The degree of inhibition of glutathione peroxidase was directly related to increased mechanical fragility with menadione, gentisic acid and potassium chlorate. However other substances causing increased mechanical fragility resulted in little or no loss of glutathione peroxidase activity.

4 The results show that there is no constant relationship between mechanical fragility caused by drugs, the formation of Heinz bodies and the inhibition of glutathione peroxidase. The factors contributing to oxidant drug-induced haemolysis appear to be variable and depend upon the drug concerned.

Introduction

Haemolysis may result from the direct toxic action of certain drugs and chemical substances, including antimalarials of the 8-aminoquinoline group, phenacetin, acetylsalicylic acid, sulphonamides, nitrofurantoin, 4,4-diaminodiphenylsulphone (Dapsone), acetyl-phenylhydrazine, menadione and sodium nitrite; this subject has been reviewed by Beutler (1969) and by Dausset & Contu (1969). In the presence of erythrocyte enzyme deficiency or haemoglobinopathy, there may be an excessive haemolytic reaction to such substances (Beutler, 1969). Drug-induced oxidative haemolysis is usually associated with the formation of Heinz bodies—insoluble precipitates within the erythrocyte, indicating the oxidative destruction of haemoglobin (Harley & Mauer, 1961). When erythrocytes are incubated with various haemolytic agents, low levels of hydrogen peroxide are produced, as a result of an autoxidation reaction with oxyhaemoglobin (Cohen & Hochstein, 1964, 1965; Cohen, 1966; Tudhope & Leece, 1971). It is presumed that hydrogen peroxide is also formed *in vivo* following the ingestion of this type of

haemolytic drug. It is generally believed that the enzyme glutathione peroxidase is primarily responsible for the removal of small amounts of hydrogen peroxide, generated in the erythrocyte as a result of the effects of oxidant drugs (Mills, 1957; Cohen & Hochstein, 1963); however such drugs may also act as enzyme inhibitors (Buzard, Kopko & Paul, 1960; Desforages, Kalaw & Gilchrist, 1960; Shahidi & Westring, 1970; Tudhope & Leece, 1971), thus impairing the ability of the erythrocyte to respond to oxidant stress.

In the present study we have investigated the relationship between Heinz body formation, increased erythrocyte mechanical fragility and the activity of glutathione peroxidase after incubation of erythrocyte suspensions with oxidant substances.

Methods

Venous blood from healthy subjects was collected in tubes containing lithium heparin. After centrifu-

gation, the plasma and leucocyte layers were removed. The erythrocytes were washed twice in NaCl (150 mmol/l), once in glucose buffered 0.9% w/v NaCl, and then made up in the latter solution so that the haemoglobin concentration of the suspension was 100-140 g/litre. A sufficient volume of this suspension was then mixed with glucose buffered saline and, when required, 0.4 ml drug solution, to give a final haemoglobin concentration of 40 g/l in 4 ml incubation mixture. Incubations were carried out in test tubes at 37°C for 16 hours. Drugs and other substances to be incubated with erythrocyte suspensions were made up in glucose buffered saline at 10 times the required final concentration.

Glucose buffered saline was prepared from nine parts NaCl (150 mmol/l) and one part Sörensen phosphate buffer (0.1 M, pH 7.0), with the final addition of glucose, 2 g/litre.

After incubation the cells were washed twice in saline and mechanical fragility was measured by the method of Goldbloom, Fischer, Reinhold & Hsia (1953), with the following modifications. Erlenmeyer flasks of capacity 50 ml were attached to the apparatus so that the mid-points of the diameters of the flasks were 3.5 cm from the centre of the rotating axis. After incubation, 1.5 ml erythrocyte suspension was rotated in the flask with eight finely polished glass beads 3 mm in diameter, for a period of 150 min at 100 rev/minute. After rotation, 0.6 ml erythrocyte suspension was added to each 1.6 ml isotonic saline and 1.6 ml distilled water for haemolysis measurements. The sample for spontaneous haemolysis was prepared by adding 0.6 ml unrotated suspension to 1.6 ml isotonic saline. Haemoglobin measurements on the three samples were made after centrifugation at 10,000 g for 10 min, by

adding 0.5 ml supernatant to 2.5 ml Drabkin's solution.

The activity of erythrocyte glutathione peroxidase (EC 1.11.1.9) was measured by a modification (Hopkins & Tudhope, 1973) of the method of Paglia & Valentine (1967), and expressed as a percentage of that of a control incubation, set up similarly except with the omission of drug. Erythrocytes were stained for Heinz bodies using crystal violet. Haemoglobin was measured spectrophotometrically as oxyhaemoglobin or cyanmethaemoglobin. The latter method was used following incubation of erythrocytes with drugs.

Results

The results of erythrocyte mechanical fragility studies, the activities of glutathione peroxidase and the counts of Heinz bodies in cells following incubation with potentially haemolytic substances are shown in Table 1. Results are shown for one concentration of each drug, incubation with which resulted in Heinz body formation in more than 20% and in less than 100% of erythrocytes. In all cases the degree of spontaneous haemolysis was less than 10%. In 43 control samples incubated with the same buffer but with no drug added, there was no Heinz body formation; the mechanical fragility ($8.6 \pm 2.7\%$) and the glutathione peroxidase activity did not differ significantly from the results with fresh unincubated cells.

Each compound was studied over a range of concentrations increasing from 0.1 mmol/l (except cupric sulphate, 0.05-0.2 mmol/litre). At concentrations shown in Table 1, potassium chlorate, gentisic acid, ascorbic acid, cupric sulphate and primaquine caused an increase in the mechanical

Table 1 Erythrocyte mechanical fragility, glutathione peroxidase activity and Heinz body formation following incubation with oxidant drugs. Results are the mean ($\pm$ S.D.) of four experiments

<i>Test substance; concentration in incubation (mmol/l)</i>	<i>Glutathione peroxidase (% activity of control incubation)</i>	<i>Mechanical fragility (%)</i>	<i>Heinz body formation (% of cells showing one or more Heinz bodies)</i>
Potassium chlorate (5.0)	5.2 $\pm$ 2.6	21.8 $\pm$ 3.6	64.2 $\pm$ 15.0
Gentisic acid (7.5)	40.0 $\pm$ 4.0	21.8 $\pm$ 5.5	80
Menadione (0.1)	89.0 $\pm$ 7.6	9.0 $\pm$ 2.1	45
Ascorbic acid (10.0)	78.5 $\pm$ 7.5	18.0 $\pm$ 4.1	78
Acetyl salicylic acid (11.1)	79.9 $\pm$ 5.3	11.2 $\pm$ 3.8	23.6 $\pm$ 17.0
Cupric sulphate (0.075)	83.7 $\pm$ 2.8	18.4 $\pm$ 3.6	98
Acetyl phenylhydrazine (2.0)	100	10.1 $\pm$ 2.8	78
Primaquine (1.0)	86.2 $\pm$ 8.4	22.9 $\pm$ 3.5	20.4 $\pm$ 10.1
Phenylhydrazine (0.1)	100	11.9 $\pm$ 3.0	13.2 $\pm$ 6.7
Salicylic acid (7.22)	95.5 $\pm$ 9.3	12.0 $\pm$ 2.8	46.0 $\pm$ 46.6

Fig. 1 The relationship between mechanical fragility of erythrocytes and the drug concentration in the incubation mixture. (Δ) Phenylhydrazine; (○) primaquine; (◻) menadione; (■) potassium chlorate; (▲) gentisic acid; (●) ascorbic acid.

fragility of the erythrocyte to a value greater than 2 SDs above the mean result with the control samples, incubated without the addition of drugs. With six substances, experiments were performed with different concentrations of the added compound and mechanical fragility increased with increasing concentrations (Figure 1). With acetylphenylhydrazine, phenylhydrazine and menadione, considerable Heinz body formation occurred at low concentrations which did not result in increased mechanical fragility. The results with phenylhydrazine are shown in Fig. 2; similar results were obtained with acetylphenylhydrazine and menadione. With primaquine, gentisic acid, potassium chlorate and ascorbic acid however, Heinz body formation occurred in parallel to increased mechanical fragility. This relationship is shown for potassium chlorate in Figure 3. Acetyl salicylic acid (ASA) and salicylic acid (SA) did not cause increased mechanical fragility at any concentration studied (up to 11.1 mmol/l with ASA and up to 7.22 mmol/l with SA).

At the concentrations stated in Table 1, marked reduction in glutathione peroxidase activity occurred only with potassium chlorate and gentisic acid. However with higher concentrations of menadione marked loss of glutathione peroxidase activity occurred; 1.0 mmol/l menadione resulted in glutathione peroxidase activity of 33% when compared to a control incubation. There was a close relationship between increased mechanical fragility and increased inhibition of glutathione peroxidase by menadione, gentisic acid and potassium chlorate. This is shown for potassium chlorate

Fig. 2 The relationship between erythrocyte mechanical fragility (■) and Heinz body formation (●) following incubation with phenylhydrazine.

Fig. 3 Erythrocyte mechanical fragility (■), Heinz body formation (●) and glutathione peroxidase (GSH-Px) inhibition (▲) following incubation with potassium chlorate.

(Figure 3). This relationship was also observed with cupric sulphate at concentrations up to 200 μ mol/litre. However with ascorbic acid and primaquine, Heinz body formation and increased mechanical fragility occurred in the presence of little or no inhibition of glutathione peroxidase.

Discussion

Changes in the osmotic fragility of red cells have been described following exposure to certain

oxidant drugs (Miller & Smith, 1970; Tudhope & Leece, 1971). Osmotic fragility measurements have the advantage of ease of reproducibility; however, the significance of an altered osmotic fragility curve in relation to the potential haemolytic effect of a drug *in vivo* is uncertain. The measurement of mechanical fragility has been used as an estimate of the susceptibility of the erythrocyte to haemolysis *in vivo* (Shen, Castle & Fleming, 1944; Earle, Bigelow, Zubrod & Kane, 1948) and it has been suggested that the testing of mechanical fragility simulates closely the physiological conditions in the blood stream (Young, Izzo & Platzer, 1951). Fok & Schuboth (1960) suggested that erythrocyte mechanical fragility occurred as a result of currents in the blood set up by the rolling movement of the glass beads, and did not result from a grinding action of the beads. The results of studies of erythrocytes from glucose-6-phosphate dehydrogenase-deficient subjects (Fraser & Vesell, 1968) indicated that mechanical fragility might be the most suitable model for the study of haemolysis produced *in vivo* by drugs.

In the present study, increased mechanical fragility occurred with all the substances studied, with the exception of salicylic acid and acetyl salicylic acid. However, gentisic acid, a metabolite of both these substances, produced a marked increase in mechanical fragility in addition to greatly reducing the activity of glutathione peroxidase and causing Heinz body formation. Gentisic acid has been shown, in our laboratory, to generate hydrogen peroxide with oxyhaemoglobin (Hopkins, unpublished observations). Shahidi & Westring (1970) found that it caused inhibition of glucose-6-phosphate dehydrogenase. It appears that inhibition of glutathione peroxidase by gentisic acid may also play a part in the haemolytic effect of acetyl salicylic acid. The present findings indicate the need for further clinical studies on the influence of metabolites of aspirin on the red cell *in vivo*.

Menadione, which causes hydrogen peroxide formation in the presence of oxyhaemoglobin (Cohen & Hochstein, 1964), also inhibited glutathione peroxidase.

This compound has been shown to cause haemolysis in infants (Allison, 1955; Meyer & Angus, 1956) and in vitamin E-deficient rats (Allison, Moore & Sharman, 1956). In the present study copper sulphate produced Heinz bodies, and increased mechanical fragility. Metz & Sagone (1972) found copper to inhibit glucose-6-phosphate dehydrogenase and GSH-reductase, and regarded copper as exerting an oxidant stress on the erythrocyte. Boulard, Blume & Beutler (1972) also found inhibition of a number of erythrocyte enzymes by copper.

Erythrocytes containing Heinz bodies show an increased tendency to haemolysis *in vivo*. Two processes may be involved (Necheles & Allen, 1969)—splenic entrapment of the more rigid, Heinz body containing erythrocytes, and lysis resulting from membrane changes consequent upon the intracellular Heinz bodies. In this study there was a variable relationship between Heinz body formation and increased mechanical fragility. With some oxidant substances, these two parameters increased in parallel, whilst in others Heinz body formation occurred prior to increased mechanical fragility. The formation of Heinz bodies by substances which caused little inhibition of glutathione peroxidase probably reflects the inability of the enzymes of the hexose monophosphate and glutathione peroxidase pathways to generate sufficient reducing potential in the face of considerable oxidant stress. It is of interest that different patterns of oxidant drug effects occur in the red cell. The present studies have shown that there is no consistent relationship between mechanical fragility, glutathione peroxidase inhibition and Heinz body formation, the relative intensity of these effects varying with different drugs. *In vivo* studies will be required to determine the significance of these findings in relation to clinical drug-induced haemolysis.

The technical assistance of Miss Joan Smith is gratefully acknowledged. This research programme was supported by a grant from the Scottish Hospital Endowments Research Trust.

References

- ALLISON, A.C. (1955). Letter, *Lancet*, i, 669.
- ALLISON, A.C., MOORE, T. & SHARMAN, I.M. (1956). Haemolysis and haemoglobinuria in vitamin E-deficient rats after injections of vitamin K substitutes. *Br. J. Haemat.*, 2, 197-204.
- BEUTLER, E. (1969). Drug induced hemolytic anaemia. *Pharmac. Rev.*, 21, 73-103.
- BOULARD, M., BLUME, K.G. & BEUTLER, E. (1972). The effect of copper on red cell enzyme activities. *J. clin. Invest.*, 51, 459-461.
- BUZARD, J.A., KOPKO, F. & PAUL, M.F. (1960). Inhibition of glutathione reductase by nitrofurantoin. *J. Lab. clin. Med.*, 56, 884-890.
- COHEN, G. & HOCHSTEIN, P. (1963). Glutathione

- peroxidase: the primary agent for the elimination of hydrogen peroxide in erythrocytes. *Biochemistry*, 2, 1420-1427.
- COHEN, G. & HOCHSTEIN, P. (1964). Generation of hydrogen peroxide in erythrocytes by hemolytic agents. *Biochemistry*, 3, 895-900.
- COHEN, G. & HOCHSTEIN, P. (1965). *In vivo* generation of H_2O_2 in mouse erythrocytes by hemolytic agents. *J. Pharmac. exp. Ther.*, 147, 139-143.
- COHEN, G. (1966). On the generation of hydrogen peroxide in erythrocytes by acetylphenylhydrazine. *Biochem. Pharmac.*, 15, 1775-1782.
- DAUSSET, J. & CONTU, L. (1969). Drug-induced hemolysis. *Ann. Rev. Med.*, 18, 55-70.
- DESFORGES, J.F., KALAW, E. & GILCHRIST, P. (1960). Inhibition of glucose-6-phosphate dehydrogenase by hemolysis inducing drugs. *J. Lab. clin. Med.*, 55, 757-766.
- EARLE, D.P., BIGELOW, F.S., ZUBROD, C.G. & KANE, C.A. (1948). Studies on the chemotherapy of the human malaras. 9. Effect of pamaquine on the blood cells of man. *J. clin. Invest.*, 27, 121-129.
- FOK, F.P. & SCHUBOTHE, H. (1960). Studies on various factors influencing mechanical haemolysis of human erythrocytes. *Br. J. Haemat.*, 6, 355-361.
- FRASER, I.M. & VESELL, E.S. (1968). Effects of drugs and drug metabolites on erythrocytes from normal and glucose-6-phosphate dehydrogenase-deficient individuals. *Ann. N.Y. Acad. Sci.*, 151, 777-794.
- GOLDBLOOM, R.B., FISCHER, E., REINHOLD, J. & HSIA, D.Y.Y. (1953). Studies on the mechanical fragility of erythrocytes. I. Normal values for infants and children. *Blood*, 8, 165-169.
- HARLEY, J.D. & MAUER, A.M. (1961). Studies on the formation of Heinz bodies. II. The nature and significance of Heinz bodies. *Blood*, 17, 418-433.
- HOPKINS, J. & TUDHOPE, G.R. (1973). Glutathione peroxidase in human red cells in health and disease. *Br. J. Haemat.*, 25, 563-575.
- METZ, E.N. & SAGONE, A.L. (1972). The effect of copper on the erythrocyte hexose monophosphate shunt pathway. *J. Lab. clin. Med.*, 80, 405-413.
- MEYER, T.C. & ANGUS, J. (1956). The effect of large doses of 'synkavit' in the newborn. *Arch. Dis. Child.*, 31, 212-215.
- MILLER, A. & SMITH, H.C. (1970). The intracellular and membrane effects of oxidant agents on normal red cells. *Br. J. Haemat.*, 19, 417-428.
- MILLS, G.C. (1957). Hemoglobin catabolism. I. Glutathione peroxidase, an erythrocyte enzyme which protects hemoglobin from oxidative breakdown. *J. biol. Chem.*, 229, 189-197.
- NECHELLES, T.F. & ALLEN, D.M. (1969). Heinz-body anemias. *New Engl. J. Med.*, 280, 203-205.
- PAGLIA, D.E. & VALENTINE, W.N. (1967). Studies on the quantitative and qualitative characterization of glutathione peroxidase. *J. Lab. clin. Med.*, 70, 158-169.
- SHAHIDI, N.T. & WESTRING, D.W. (1970). Acetylsalicylic acid-induced hemolysis and its mechanism. *J. clin. Invest.*, 49, 1334-1340.
- SHEN, S.C., CASTLE, W.B. & FLEMING, E.M. (1944). Experimental and clinical observations on increased mechanical fragility of erythrocytes. *Science, N.Y.*, 100, 387-389.
- TUDHOPE, G.R. & LEECE, S.P. (1971). Red cell catalase and the production of methaemoglobin, Heinz bodies and changes in osmotic fragility due to drugs. *Acta haemat.*, 45, 290-302.
- YOUNG, L.E., IZZO, M.J. & PLATZER, R.F. (1951). Hereditary spherocytosis. I. Clinical, hematologic and genetic features in 28 cases, with particular reference to the osmotic and mechanical fragility of incubated erythrocytes. *Blood*, 6, 1073-1097.

(Received September 25, 1973)