Some Effects of Chlorambucil on Enzymes of Glutathione Metabolism in Drug-Sensitive and -Resistant Strains of the Yoshida Ascites Sarcoma

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1. Yoshida ascites cells from a strain sensitive to chemotherapy with alkylating agents contained elevated activities of the two enzymes directly responsible for glutathione synthesis, in comparison with a resistant cell strain. The activities of the glutathione-degrading enzyme γ -glutamyltranspeptidase were comparable in both cell strains. 2. After parenteral administration of chlorambucil to rats carrying either strain of tumour, the activities of the glutathione-synthetic enzymes increased in the sensitive cells, but remained unchanged in the resistant cells. Drug treatment was without effect on the γ -glutamyltranspeptidase activity of either cell strain. 3. The activities of a number of enzymes concerned in the oxidoreduction of glutathione remained unchanged after the administration of chlorambucil to rats carrying either strain of cells.

In previous papers it has been shown that GSH accumulated in the cytoplasm of a sensitive strain of Yoshida ascites-sarcoma cells when the host animals were treated with alkylating agents, that comparable effects were not obtained in a strain of the tumour that was refractory to chemotherapy, that both qualitative and quantitative differences in GSH accumulation were observed according to the identity of the drug administered, and that chlorambucil treatment resulted in the appearance of GSSG at 4 hr. in sensitive cells and at 28 hr. in resistant cells (Harrap & Hill, 1968*a,b*). Since this drug-induced elevation in the GSH content occurred only in the sensitive cells, it was possible that the effect might be related to the mechanism of drug-resistance. Further, this experimental system provided a convenient model with which to study the control of intracellular GSH contents.

The relationships between a number of GSH-metabolizing enzymes are illustrated in Scheme 1. Synthesis is catalysed by (1 in Scheme 1) γ -glutamylcysteine synthetase (EC 6.3.2.2) and (2) GSH synthetase (EC 6.3.2.3), and degradation of GSH is catalysed by (3) γ -glutamyltranspeptidase (EC 2.3.2.-). GSH oxidation is catalysed by (4) GSH peroxidase (EC 1.11.1.9), and reduction of GSSG is catalysed by (5) GSSG reductase (EC 1.6.4.2). Of the enzymes catalysing the reduction of NADP, (6) glucose 6-phosphate dehydrogenase (EC 1.1.1.49), (7) 6-phosphogluconate dehydrogenase (EC 1.1.1.43) and (8) isocitrate dehydrogenase (NADP) (EC 1.1.1.42) were selected for study.

The work described in this paper investigated

the possibility that the chlorambucil-induced accumulation of GSH and GSSG, described above, resulted from a modification in the content of one or more of the apoenzymes shown in Scheme 1.

MATERIALS AND METHODS

Chlorambucil, 3-(*p*-[bis-(2-chloroethyl)amino]phenyl)-L-alanine, and melphalan, 4-(*p*-[bis-(2-chloroethyl)amino]phenyl)butyric acid, were synthesized in the Chester Beatty Research Institute. Reagent chemicals were obtained from Hopkin and Williams Ltd. (Chadwell Heath, Essex) and British Drug Houses Ltd. (Poole, Dorset), A.R. grades being used where available. GSSG and GSH were purchased from Sigma Chemical Co. (St Louis, Mo., U.S.A.) and glucose 6-phosphate, 6-phosphogluconate, NADPH₂ and ATP from Boehringer Corp. (London) Ltd. (London, W. 5). Glyoxylic acid monohydrate was supplied by Koch-Light Laboratories Ltd. (Colnbrook, Bucks.) and 5,5'-dithiobis-(2-nitrobenzoic acid) by Aldrich Chemical Co. Inc. (Milwaukee, Wis., U.S.A.). γ -Glutamylcysteine was prepared by the method of Sheehan & Yang (1958).

Female Wistar rats of the Chester Beatty strain were used at 6 weeks of age, having body weights approx. 200 g. The tumour was passaged by intraperitoneal injection of 2×10^6 cells in aq. 0.9% NaCl containing 2000 units each of benzylpenicillin and streptomycin. The resistant line was maintained by treatment, 3 days after each routine passage, with 4 mg. of melphalan/kg. body wt.: this operation was omitted in those animals that were to be used subsequently for experimental purposes.

Chlorambucil was dissolved in the minimum quantity of a 2% (w/v) soln. of HCl in ethanol and diluted to the required concentration with a phosphate-propylene glycol buffer, pH 8.5 (20 g. of KH₂PO₄ + 450 ml. of propylene glycol, diluted to 1 l. with water). The drug was administered subcutaneously at a dose of 8 mg./kg. body wt. on the fifth

Scheme 1. Some enzymes catalysing the synthesis, degradation and oxidoreduction of GSH. The numbered enzymes are defined in the text.

day after tumour transplantation. This dose was sufficient to induce complete regression of the sensitive tumour, but was without effect on the growth rate of the resistant tumour. Control animals received solvent only. Untreated animals and control animals (carrying either the sensitive or the resistant tumour) died on the ninth or tenth day after tumour transplantation. Animals were killed (usually in groups of three) by cervical dislocation at various times after drug treatment, and the peritoneal contents were aspirated with ice-cold aq. 0.3% NaCl solution: all subsequent preparative manipulations were performed at 0°. The cells were collected by centrifugation at 350g for 10 min., and any erythrocytes remaining in the pellet were removed by 'osmotic shock' (Walford, Peterson & Doyle, 1957). The tumour cells were washed in aq. 0.9% NaCl solution and resuspended to a known volume, also in aq. 0.9% NaCl solution, to provide the 'tumour-cell preparation'. Cell concentration in this suspension was determined with an electronic particle counter (model A; Coulter Electronics, Kenmore, Chicago, Ill., U.S.A.) with threshold and aperture current settings 15 and 2 respectively.

Enzyme assays. Cell lysates were prepared by ultrasonic treatment of samples of the 'tumour cell preparation' at 20 kcyc./sec. (MSE Ultrasonicator) for 2 min. The 'whole lysate' was used in the assay of γ -glutamylcysteine synthetase, GSH synthetase and γ -glutamyltranspeptidase, and the remaining enzymes were assayed in a 'supernatant fraction', prepared by centrifuging the 'whole lysate' at 20000g for 20 min. γ -Glutamylcysteine synthetase (1 in Scheme 1), GSH synthetase (2) and γ -glutamyltranspeptidase (3) were assayed by the methods described by Jackson (1969), with the following modifications. (1) For the incubation medium all reagent volumes were decreased by half, 0.5 ml. of 'whole lysate' (from 10^8 cells) was added, and the final volume was adjusted to 2.0 ml. with water. Colour development was achieved with 0.25 ml. of 3.8 mm-5,5'-dithiobis-(2-nitrobenzoic acid) in 1.0 M-phosphate buffer, pH 6.8, and E_{410} was measured in a 1 cm.-light-path cell after 10 min. (2) 'Whole lysate' (1 ml. from 10^8 cells) was added to the incubation mixture, and the volume was

adjusted to 4 ml. with water. The mixture was incubated at 37° for 120 min. (3) 'Whole lysate' (0.1 ml. from 2×10^7 cells) was added to the incubation mixture, and the volume was diluted to 1 ml. with water. The mixture was incubated anaerobically for 60 min.

GSH peroxidase (4) was assayed by the method of Mills (1959), as modified by Harrap (1965), and GSSG reductase (5) by the method of Scott, Duncan & Ekstrand (1963). Glucose 6-phosphate dehydrogenase (6) and 6-phosphogluconate dehydrogenase (7) were assayed by the method of Shonk & Boxer (1964). Isocitrate dehydrogenase (8) was measured by the method of Siebert, Carsiotis & Plant (1957).

Unit of enzyme activity. This was defined as the number of μ moles of substrate converted/ 10^9 cells/min. at 0° for GSH peroxidase, at 37° for the GSH-synthetic and degradative enzymes, and at 25° for the remaining enzymes. Human erythrocytes were obtained and treated by the procedure described by Jackson (1969).

RESULTS

First we investigated the possibility that chlorambucil might inhibit (presumably by alkylation) the enzymes responsible for GSH synthesis or degradation. Table 1 indicates the effects of incubation of chlorambucil *in vitro* on the activity of γ -glutamylcysteine synthetase and GSH synthetase from human erythrocytes, and on the activity of ascitic γ -glutamyltranspeptidase. (The GSH-synthetic pathway in erythrocytes is more active than in ascites cells, and represents a convenient source for inhibition studies. Under the experimental conditions adopted, no interference of GSH synthesis by the transpeptidase enzyme was encountered.) Both synthetic enzymes were inhibited, but chlorambucil was without effect on the activity of γ -glutamyltranspeptidase.

Table 1. Effect of chlorambucil on the activity of γ -glutamylcysteine synthetase, GSH synthetase and γ -glutamyltranspeptidase *in vitro*

For experimental details, see the text.

Enzyme	Concn. of chlorambucil (μ g./ml. of incubation mixture)	Relative activity
γ -Glutamylcysteine synthetase	Nil	100
	3.7	88
	7.5	69
GSH synthetase	Nil	100
	3.7	93
	7.5	82
γ -Glutamyltranspeptidase	Nil	100
	2.5	96
	5.0	95
	7.5	97

Fig. 1. Effect of parenteral administration of chlorambucil on the activity of γ -glutamylcysteine synthetase in ascites cells. $\circ$, Sensitive treated; $\bullet$, sensitive control; $\square$, resistant treated; $\blacksquare$, resistant control. Each point represents mean of determinations from at least three individual animals. The overall scatter about any point $\pm 16\%$.

Figs. 1 and 2 show the effects of parenteral administration of chlorambucil on the activities of γ -glutamylcysteine synthetase and GSH synthetase respectively in the ascites cells. Commencing 24 hr. after injection, the activities of both enzymes increased in the sensitive cells of treated animals, until at 50 hr. the activities were approximately 50% greater than in the corresponding 'control' cells. No alteration in enzyme activity occurred in the resistant cells. It is noteworthy that the activities of γ -glutamylcysteine synthetase and of GSH synthetase in untreated sensitive cells were 100% and 30% greater respectively than in untreated resistant cells. Chlorambucil treatment was without effect on the γ -glutamyltranspeptidase activity

Fig. 2. Effect of parenteral administration of chlorambucil on the activity of GSH synthetase in ascites cells. $\circ$, Sensitive treated; $\bullet$, sensitive control; $\square$, resistant treated; $\blacksquare$, resistant control. Each point represents mean of determinations from at least three individual animals. The overall scatter about any point $\pm 12\%$.

(3.8 units/ 10^9 cells) of cells from either tumour strain up to 50 hr. after treatment.

The effects of chlorambucil on the activities of apoenzymes of the GSH oxidation-reduction pathway are summarized in Table 2. 'Resistant' cells were removed at 24 hr. and 'sensitive' cells at 4 hr. after treatment, since at these times the GSSG contents were at a maximum (Harrap & Hill, 1969b). The drug did not lower the contents of the reducing enzymes, neither was the GSH peroxidase activity raised in either cell strain. With the exception of glucose 6-phosphate dehydrogenase the activities of all enzymes were broadly comparable in untreated cells of either strain.

DISCUSSION

The concentration of chlorambucil required to inhibit the GSH-synthetic enzymes *in vitro* could not have been achieved in the ascites cells *in vivo* after the administration of 8 mg. of drug/kg. body wt., assuming a uniform distribution of the drug throughout the animal. This inhibition may therefore be disregarded when one is considering the results of the studies *in vivo*.

The activities of the GSH-synthetic enzymes were greater in lysates of sensitive ascites cells than in those of resistant cells: in response to chlorambucil treatment the activities of both these enzymes increased to the same extent in sensitive cells, whereas no change was observed in resistant cells. On the other hand, the γ -glutamyltranspeptidase activities of both cell strains were comparable, and were unaffected by chlorambucil treatment. In other words, it seemed that the sensitive cell strain had a greater potential for GSH synthesis, which was increased by drug treatment, whereas the capacity for GSH degradation was comparable in

Table 2. *Mean activities of enzymes of the GSH oxidoreduction pathway in ascites cells from control and chlorambucil-treated animals*

The values represent means of duplicate samples from three separate animals. The numbers in parentheses give the ranges of values obtained.

Enzyme ...	Activity (μ moles of substrate converted/ 10^8 cells/min.)				
	GSSG reductase	Glucose 6-phosphate dehydrogenase	6-Phosphogluconate dehydrogenase	Isocitrate dehydrogenase	GSH peroxidase
Resistant (24 hr.)					
Control	2.9 (2.8-2.9)	9.0 (8.4-9.5)	2.9 (2.7-3.0)	7.8 (6.6-8.9)	2.1 (1.9-2.3)
Treated	2.8 (2.6-3.2)	11.1 (8.8-12.5)	2.9 (2.5-3.2)	7.3 (7.2-7.4)	2.8 (2.4-3.1)
Sensitive (4 hr.)					
Control	3.1 (2.8-3.5)	4.8 (4.3-5.1)	4.3 (3.6-5.3)	6.5 (5.4-7.3)	2.8 (2.6-3.0)
Treated	3.6 (3.4-4.1)	5.9 (5.6-6.4)	5.0 (4.1-5.7)	6.7 (5.8-7.3)	2.3 (1.9-2.7)

both cell strains and was unaffected by treatment. This may represent one important factor in the increase in GSH that occurred in the sensitive cells in response to chlorambucil therapy.

Chlorambucil treatment did not limit the activity of the enzymes of the GSH oxidoreduction system, and it was not possible to attribute the formation of GSSG in either cell strain to an impairment of any of the component apoenzymes of this system. Although the integrity of the apoenzymes has been established, it is still possible that a decrease in holoenzyme function could have resulted from a lowered cofactor concentration. It is known that administration of alkylating agents can result in a lowering of the concentration of NAD *in vivo* (Kun, Langer, Ulrich, Holzer & Grunicke, 1964), and Green & Bodansky (1962) showed that the NAD content of Ehrlich ascites cells fell in response to parenteral treatment of the host animals with 1,3-bis-(2-chloroethyl)-1-nitrosourea. It is not known what effects such treatment would have on the overall proportions of the nicotinamide nucleotide cofactors present in the experimental system discussed here.

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