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SOME EFFECTS OF CHLORAMBUCIL ON THE UPTAKE AND UTILIZATION OF SULPHYDRYL-CONTAINING COMPOUNDS BY TUMOUR CELLS

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Abstract—The alkylating drug chlorambucil is without effect on the rate of uptake of cysteine or glutathione in two strains of Yoshida ascites cells (differing 100-fold in sensitivity to the drug). The pools of low molecular weight thiols were not altered by the drug, neither did it cause the release of these components from combination with mixed disulphide. It is concluded that, in this system, there is no support for the speculation that acquired resistance to alkylating agents can be attributed to elevated pools of low molecular weight thiols, or to their release from mixed disulphide linkage.

DRUG RESISTANCE is a persisting problem in the treatment of neoplastic disease, and several mechanisms have been proposed to account for this phenomenon in the case of the alkylating agents. There is good evidence that both microorganisms and mammalian cells in culture can excise and repair alkylated DNA.[1,2] Other workers have noted an impaired uptake of alkylating agents by resistant cells,[3] while the ability of such cells to degrade certain alkylating drugs has also been observed.[4,5] It has been suggested frequently that one mechanism responsible for drug resistance is the interaction of alkylating agents with thiol-containing compounds present in the cytoplasm of resistant cells.[6–9] The ability of cells to detoxify alkylating agents by this route is dependent not only on an adequate intracellular level of low molecular weight thiols, but also on a mechanism for the mobilization of these compounds in response to drug challenge. Factors which may be implicated in these processes are indicated schematically in Fig. 1. Intracellular levels of cysteine (Cys) are maintained by the extracellular supply and by protein catabolism, and are in equilibrium with cystine (CySSCy). Further, Cys is utilized in the synthesis of glutathione (GSH), which is also in equilibrium with its oxidized form (GSSG). Considerable quantities of Cys and GSH are bound as mixed disulphides[10] (i.e. unsymmetrical disulphides CySSR or GSSR, which on reduction release CySH or GSH and a protein thiol, RSH, which may be precipitated by trichloracetic acid). Although Modig[11] has proposed that mixed disulphides provide a reserve of thiols, it is not known to what extent mixed disulphides are able to contribute to the intracellular thiol pool.

The present communication describes the results of work designed to investigate the effects of chlorambucil on the transport of thiols across the cell membrance, and on their subcellular binding. The effects have been monitored in drug-sensitive and

drug-refractory (100-fold) lines of the Yoshida ascites sarcoma[12] in order to assess their relevance to the development of acquired resistance.

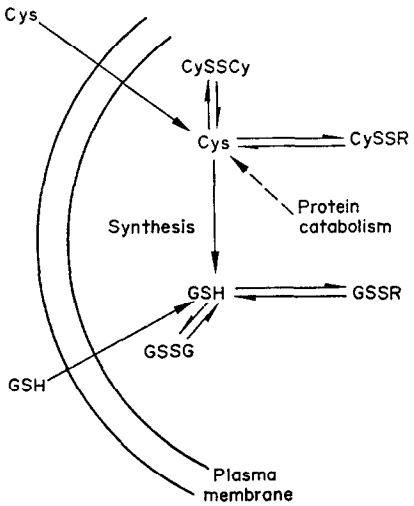

FIG. 1. The transport of thiols across the cell membrane, and their intracellular mobilisation. Cys, cysteine; CySSCy, cystine; GSH, glutathione; GSSG, oxidized glutathione; CySSR, protein-bound mixed disulphide of cysteine; GSSR, protein-bound mixed disulphide of glutathione.

MATERIALS AND METHODS

The Yoshida tumour lines were maintained by weekly passage in female Wistar rats of the CB strain (body wt approx. 200 g). 2×10^6 tumour cells of either strain were passaged by i.p. injection on day 0. For *in vitro* experiments, chlorambucil was given subcutaneously, at a dose of 8 mg/kg, on the 4th or 5th day following transplantation. The animals were killed at varying times thereafter by cervical dislocation, and the tumour cells removed by aspiration in 0·3% ice-cold phosphate buffered saline pH 7·4. More extensive accounts of these procedures appear in earlier reports from this laboratory.[13,14]

In vitro uptake studies: tumour cells were washed three times in 0·3% ice-cold phosphate buffered saline and then suspended in Hank's solution at a final concentration of 10^6/ml; neutralized solutions of ^{35}SCys and ^{35}SGSH in Hank's solution were added up to a final concentration of 1 mM (1 μCi/ml). Incubations were carried out at 37° and samples removed at timed intervals after the addition of labelled thiol. Cells were removed from 10 ml of suspension by centrifugation at 300 *g*, washed twice with Hank's solution at 0°, extracted with two 5 ml vol. of 5% trichloroacetic acid (TCA), and the protein precipitate removed by centrifugation at 2000 *g*. The residue was dissolved in 1 ml of 12·5% aqueous tetraethylammonium hydroxide (TEH). 0·1 ml vol. of the TCA-soluble supernatant or of the TEH solution were added to 10 ml of scintillation fluid (toluene 1925 ml, dioxan 1925 ml, methanol 1150 ml, naphthalene 400 *g*, butyl PBD 40 *g*) and the ^{35}S activity counted in a Packard scintillation spectrometer. Soluble and particulate cell preparations were obtained by sonication (2 min at 30 k Hz, 0°) of a cell suspension, followed by centrifugation at 25,000 *g* for 30 min (0°).

Mixed disulphide was estimated by the method of Modig as modified by Harrap *et al.*[10], and protein -SH as described by Jocelyn.[15] Protein was determined by the method of Lowry *et al.*[16]

Chlorambucil (Leukeran), $(ClCH_2CH_2)_2N.C_6H_4(CH_2)_3COOH$, was synthesized in the Chester Beatty Research Institute. ^{35}S-labelled Cys and GSH were purchased from the Radiochemical Centre, Amersham, U.K., and Schwarz Bioresearch, New York, U.S.A. respectively. Cys was obtained from B.D.H. Ltd., and GSH from Sigma Chemical Co. Reagent chemicals were purchased from Hopkin & Williams Ltd. or B.D.H. Ltd., AnalaR grades being used where available.

RESULTS

The uptake of ^{35}S-GSH and ^{35}S-Cys during 20 min by the two strains of Yoshida cell *in vitro* is illustrated in Fig. 2. Uptake of Cys and GSH occurred against a concentration gradient: after a 20 min exposure to a 1 mM concentration of ^{35}S-thiol, the intracellular concentration achieved in the sensitive cells was 3·0 mM for Cys and 1·2 mM for GSH, 0·1 mM dinitrophenol was without effect on the uptake of Cys or GSH. The difference in uptake between the two cell lines is of small significance.

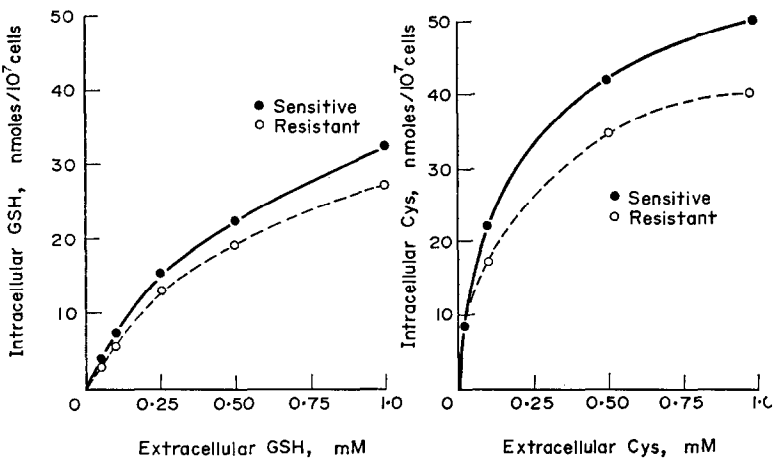

FIG. 2. Uptake of ^{35}S-Cys and ^{35}S-GSH by Yoshida ascites sarcoma cells. Cells (10^7/ml) were incubated with labelled thiol for 20 min at 37°. Intracellular thiol concentrations were calculated from the total counts incorporated. Scatter ± 10 per cent.

The rates of efflux of ^{35}S-Cys and ^{35}S-GSH from sensitive and resistant cells are compared in Fig. 3. In both cases, the thiol previously accumulated into the soluble compartment was gradually eliminated over an 8 hr period, while there was little change in the counts incorporation into protein. This behaviour was not unique to the thiol-containing derivatives, since lysine was taken up and eliminated in a similar fashion, though in this case the incorporation into protein increased over the time of reincubation (see Table 1).

Uptake of ^{35}S-Cys was unaffected when either cell type was incubated *in vitro* with 0·005 mM or 0·1 mM chlorambucil, either in presence of the amino acid, or if the cells

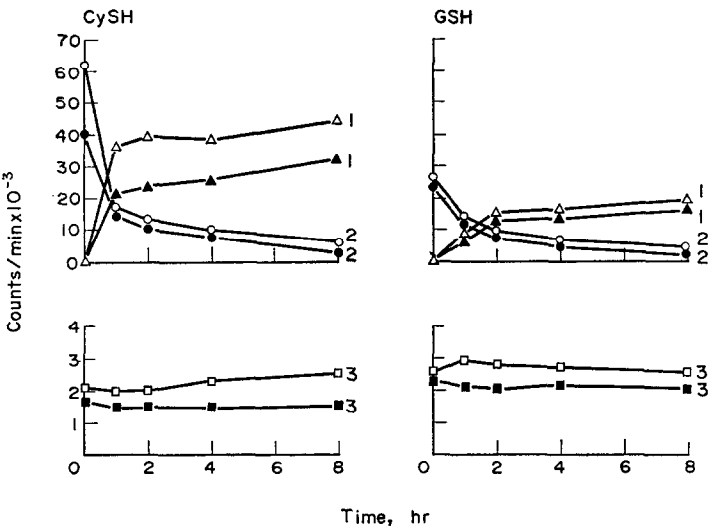

FIG. 3. Efflux of ^{35}S-Cys and ^{35}S-GSH from Yoshida ascites sarcoma cells. Cells were prelabelled for 20 min at 37° and then reincubated for the time periods indicated. (△, ○, □) Sensitive cells; (▲, ●, ■) resistant cells; (1 = ^{35}S-) in incubation medium; (2 = ^{35}S-) in TCA soluble fraction from cells; (3 = ^{35}S-) in TCA insoluble fraction from cells.

were treated first with the drug for 1 hr, washed, and then exposed to labelled Cys. Treatment of tumour-bearing animals with chlorambucil (8 mg/kg) was without effect on the subsequent rate of Cys transport *in vitro* into the resistant cells. However, uptake into the sensitive cells did increase (see Fig. 4), though this was accompanied by a proportionate increase in cell volume and protein content[13,14] so that the intracellular thiol concentration remained unchanged.

TABLE 1. THE FATE OF THE TCA-SOLUBLE AND TCA-INSOLUBLE COMPARTMENTS OF YOSHIDA ASCITES SARCOMA CELLS LABELLED WITH ^{35}S-Cys, ^{35}S-GSH OR ^{3}H-LYSINE, AFTER SUBSEQUENT INCUBATION IN UNLABELLED MEDIUM FOR 4 hr

	TCA-soluble		TCA-insoluble	
	Counts/min per 10^7 cells at zero time $\times 10^{-3}$	% counts after 4 hr	Counts/min per 10^7 cells at zero time $\times 10^{-3}$	% counts after 4 hr
Cys				
Sensitive	53·32	29	2·50	100
Resistant	44·50	32	2·64	86
GSH				
Sensitive	22·72	16·3	2·75	84
Resistant	20·08	12·5	2·69	82
Lysine				
Sensitive	93·20	3·4	5·39	240
Resistant	126·40	2·0	3·00	258

The cells were prelabelled by exposure to the labelled compound for 20 min (counts/min at zero time): they were then washed in saline and resuspended in fresh medium.

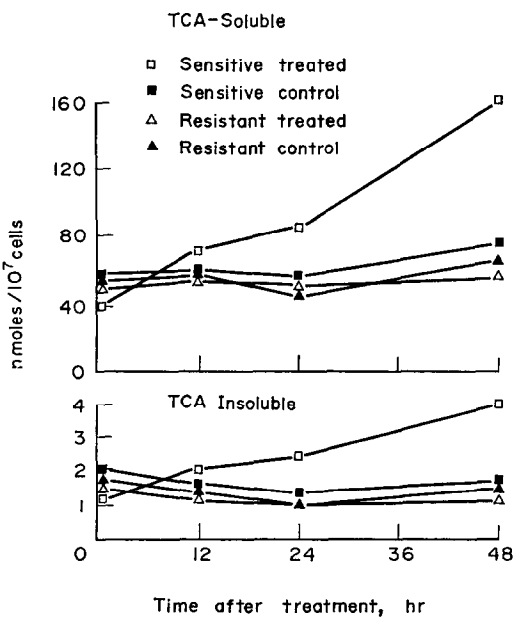

FIG. 4. Uptake of ^{35}S-Cys into TCA-soluble and insoluble fractions of Yoshida ascites sarcoma cells from animals treated with chlorambucil (8 mg/kg).

The protein-thiol content of Yoshida cells, and the levels of mixed disulphides are listed in Table 2. It can be seen that cells of either line contain almost identical amounts of these components. When tumour-bearing animals were treated with chlorambucil (8 mg/kg), the drug was without effect on the resistant cells. In the sensitive cells, however, there was an increase in the mixed disulphide and protein-thiol contents after 24 hr (see Fig. 5). However, this was accompanied by a corresponding increase in protein content and cell volume,[13,14] so that no change in the concentration of these components occurred.

TABLE 2. COMPARISON OF SOME PROPERTIES OF THE SENSITIVE AND RESISTANT STRAINS OF YOSHIDA SARCOMA BEFORE TREATMENT. VALUES QUOTED ARE MEAN, AND RANGE, OF FOUR DETERMINATIONS

	Sensitive	Resistant
Protein content of 25,000 *g* (30 min) supernatant (mg/10⁹ cells)	86 (66–114)	89 (75–99)
Protein-SH μ-equiv./10⁹ cells	5·8 (5·4–6·3)	5·7 (4·7–6·3)
Total mixed disulphide μ-equiv./10⁹ cells	7·9 (7·2–8·6)	8·0 (4·1–10·9)
Protein-S.S.G. as % of total mixed disulphide	58	66

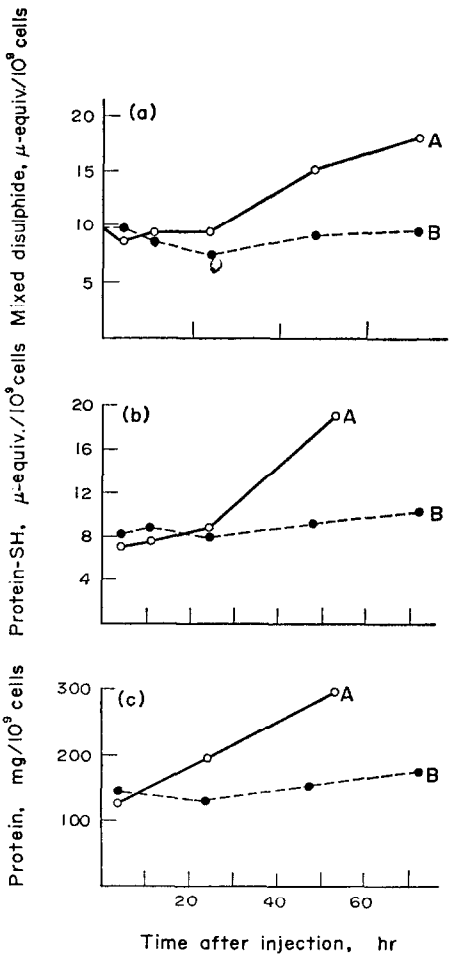

FIG. 5. Mixed disulphide (5a), protein SH (5b) and protein (5c) in sensitive Yoshida ascites cells following chlorambucil treatment. A = treated; B = control.

The stability of the protein mixed disulphide pools was compared in the two cell lines following treatment with chlorambucil as follows: animals bearing either sensitive or resistant tumours received 26·5 μCi of ^{35}S-Cys i.p., Half the animals received, in addition, chlorambucil (8 mg/kg.s.c.). The tumours were harvested 18 hr later, and the washed cells extracted with 10% TCA to give an acid-soluble fraction, while the residue was extracted with ethanol, then ether, and finally dried. Weighed amounts of the TCA-insoluble material were counted directly, and also reduced with sodium borohydride to obtain an estimate of the mixed disulphide content. The results were closely comparable for both cell types, and were not changed by the treatment: ^{35}S-Cys associated with acid soluble fraction = 45 per cent; ^{35}S-Cys bound as MDS in the acid insoluble fraction = 15 per cent; ^{35}S-Cys incorporated into acid pricipitate protein 40 per cent. The turnover of the protein-bound mixed disulphide pools was assessed in the following experiment: 0·1 mCi ^{35}S-Cys was added to a 500 ml culture (10^5 cells/ml) of Yoshida cells growing in Fischers medium for leukaemic cells of mice

TABLE 3. TURNOVER OF ^{35}S-Cys PREVIOUSLY INCORPORATED INTO MIXED DISULPHIDE AND PROTEIN

Cells	Time after labelling (hr)	Counts/min × 10^{-3} per mg protein							
		25,000 *g* supernatant				25,000 *g* pellet			
		^{35}S-Cys in MDS		^{35}S-Cys in protein		^{35}S-Cys in MDS		^{35}S-Cys in protein	
		Control	Treated	Control	Treated	Control	Treated	Control	Treated
Sensitive	12	82	78	73	53	92	94	98	77
	24	93	80	44	35	93	72	63	54
Resistant	12	122	140	68	81	103	114	106	77
	24	75	79	36	42	68	56	47	58

Figures quoted are the means of duplicate estimations (scatter ± 20 per cent).

(containing 20 per cent foetal calf serum). After 12 hr the cells were washed and resedimented (at 350 *g*) three times with fresh medium, and set up again at 10^5 cells/ml in fresh medium. They were then exposed to 5×10^{-6}M chlorambucil. At 12 and 24 hr thereafter, 10^6 cells were separated by sedimentation at 350 *g*, washed three times with phosphate buffered saline pH 7·4, resuspended in 10 ml phosphate buffered saline pH 7·4, sonicated at 30 kHz, dialysed for 16 hr against the same buffer, and the dialysed preparation centrifuged at 25,000 *g* for 30 min. The counts bound to mixed disulphide and to protein in the soluble and particulate preparations were then measured as outlined in the experimental section. The results are summarized in Table 3. The mixed disulphide content of the supernatant and pellet preparations from sensitive cells was stable over the period of observations, and was unchanged by drug treatment. In the resistant cells however, the mixed disulphide content of the soluble and particulate preparations fell by approximately 40 per cent, but this loss was not influenced by drug treatment. ^{35}S-Cys bound to protein also decreased by about 40 per cent in both preparations from each cell strain, and this decrease remained unaffected by treatment.

DISCUSSION

The rates of influx and efflux of Cys and GSH in two strains of the Yoshida tumour are closely comparable. Cys uptake is not inhibited by dinitrophenol, yet takes place against a concentration gradient, and probably occurs, therefore, by facilitated diffusion. Chlorambucil was without effect on the transport of Cys into cells of either strain: the enhanced uptake of the thiol seen in sensitive cells harvested from treated animals correlates with the concomitant increase in protein and cell volume observed previously.[13,14] There was no evidence that resistant cells contained higher pools of low-molecular weight thiols, or that these pools were influenced by drug treatment.

It is also apparent in these cells that small molecular weight thiols are not liberated from mixed disulphides in response to contact with alkylating agents. Neither does this source provide a reserve of Cys for protein synthesis, since no movement of ^{35}S from mixed disulphide into protein was detected in turnover measurements.

Our overall conclusion is that there is no evidence, in the system studied here, to support the frequently raised speculation[6–9] that alkylating agent resistance is attributable to high intracellular thiol levels, or to the presence of disulphides from which thiols may be released readily.

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