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STUDIES ON THE TRANSPORT AND CELLULAR DISTRIBUTION OF CHLORAMBUCIL IN THE YOSHIDA ASCITES SARCOMA

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Abstract—Previous reports[1,2] indicating that tritium-labelled chlorambucil is rapidly taken up by drug-sensitive and drug-resistant Yoshida ascites sarcoma cells *in vitro* have been confirmed. Experimental evidence indicates that chlorambucil enters the cells by passive diffusion: the rate and extent of uptake is independent of temperature, is not inhibited by fluoride, cyanide, 2-4-dinitrophenol, iodoacetate or an ATPase inhibitor, ouabain, and uptake does not occur against a concentration gradient. Inactive analogues of chlorambucil, for example hydrolysed chlorambucil *N*-bis(dihydroxyethyl)phenyl-butyric acid is incorporated less effectively by these cells than the parent compound.

The observed 2-fold difference in gross uptake by the two cell strains at 37°[1] is not apparent at 4°. This reduced ability of resistant cells to take up chlorambucil is therefore not due to an impairment of the transport mechanism, but may be related to their enhanced ability to metabolize the drug at 37°.

The extent of drug binding to cellular macromolecular components is considerably reduced at low temperatures.

NUMEROUS mechanisms have been proposed in an attempt to explain the development of resistance to alkylating agent treatment, but little direct evidence is available in favour of any one of them. Our own initial studies *in vitro* have demonstrated: a decreased uptake of [^{3}H]chlorambucil by a drug-resistant strain of the Yoshida ascites sarcoma;[1] and differences in the utilization of chlorambucil by drug-resistant and drug-sensitive strains of the tumour.[2]

The present study was designed to obtain more information on the following questions: how is the drug transported into the cell and can the differences in gross uptake by the two cell strains be attributable to any specific alteration of this transport process?; is this decreased uptake of drug by resistant cells attended by differences in binding to nucleic acids and proteins?

MATERIALS AND METHODS

Tritium-labelled chlorambucil 4-(4-di-(2-chloroethyl)amino-3,5-[^{3}H]phenyl)butyric acid was synthesized by the reductive tritiation of the iodinated derivative in the Chester Beatty Research Institute,[3] where hydrolysed chlorambucil [*N*-bis(dihydroxy-ethyl) phenyl-butyric acid] was also prepared. [^{14}C]inulin carboxylic acid (specific activity 8·6 mc/m-mole) was obtained from The Radiochemical Centre, Amersham. Other chemicals were purchased from Hopkin and Williams Ltd., or BDH. Ltd., AnalaR grades being used where available.

Animals

Full details of animal experimentation and tumour transplantation techniques have been described previously.[4,5] Animals were killed by cervical dislocation on the 5th day after tumour transplantation. The peritoneal contents were aspirated with ice-cold 0·3% saline, washed in a solution of phosphate-buffered saline (PBS) and resuspended to a known volume. The cell concentration was determined in an electronic particle counter, Model A (Coulter Electronics, Kenmore, Chicago) with threshold and aperture current settings 15 and 2 respectively.

Cell suspensions

10^8 cells were suspended in 9·9 ml of PBS, and drug solutions, or solvent alone (EtOH), were added in 0·1-ml volumes. Incubations were carried out in a metabolic shaker (Gallenkamp).

Drug uptake

Drug uptake was followed by two methods: (1) a technique involving the use of glass fibre discs. Duplicate 1 ml samples were removed from the incubation mixture at various time intervals after drug addition (from 30 sec to 1 hr), and pipetted separately onto a Millipore filter containing a glass fibre disc (Whatman type GF/C). Suction was applied and the disc was washed thrice with 5-ml vol. of ice-cold PBS to remove any ^{3}H-labelled chlorambucil which had not been taken into the cells. The disc was air-dried by suction and then placed in a glass vial and allowed to dry overnight at 50°, to which was then added 10 ml of scintillator.

(2) by withdrawing 2 ml aliquots of cell suspension at measured time intervals after drug addition (from 5 min to 2 hr). Cells were removed by centrifugation at 500 *g* (4°), and washed twice with 5-ml vol. of ice-cold PBS. The resultant cell pellets were then either: (a) dissolved in 12·5% aqueous tetraethylammonium hydroxide (TEH) for measurement of gross drug uptake by the cells or,

(b) extracted by shaking for 1 min successively with 2 × 2 ml volumes of ethanol (4°), allowed to stand at 0° for 30 min before removing and keeping the ethanol-supernatant fraction and solubilizing the ethanol-insoluble fraction in 1 ml NaOH or,

(c) subjected to the Kirby procedure[6] to obtain DNA, RNA and protein containing fractions.

Aliquots of these extracts were assayed for radioactivity using a Toluene–Phosphor scintillant in a Packard Tri-Carb Liquid Scintillation Counter Model 3375.

DNA was estimated according to Burton,[7] RNA by the orcinol procedure[8] and protein by the method of Lowry *et al.*[9]

Drug metabolism studies

The metabolism of chlorambucil and its dihydroxyderivative were followed by the method described previously utilizing optical density determinations at 258 mμ.[2]

Determination of the volume of cell water in ascites cells

The technique used was a modification of the method of Goldman *et al.*[10] An aliquot of cell suspension containing 5 × 10^7 cells was centrifuged at 500 *g* for 15 min

and the supernatant liquid removed. The resultant cell pellet was resuspended in 2 ml [^{14}C]inulin carboxylic acid in saline to a final concentration of 0·04 μc/ml. After equilibration at room temperature for 15 min, 0·4-ml aliquots of the cell suspension were transferred to previously weighed micro polypropylene tubes and centrifuged at 1300 *g* for 15 min. The supernatant was removed with a syringe and the tubes were drained for 5 min. 0·1-ml aliquots of the supernatant were taken for scintillation counting. The tube was then reweighed to determine the wet weight of the cell pellet and the dry weight was obtained by heating the tube to constant weight at 65°. Finally the cell pellets were dissolved in 0·5 ml of 12·5% aqueous TEH and 0·2 ml samples were assayed for radioactivity by scintillation counting.

By this technique it was possible to calculate the extracellular water, wet weight and dry weight of each cell pellet and thus to determine the intracellular water.

The total chlorambucil present in cell extracts obtained from 5×10^7 cells was determined and the concentration of drug present in cell water was calculated. The decrease in the concentration of chlorambucil in the medium due to its uptake by the cells was neglected since it was very small compared to the concentration of chlorambucil in the incubation medium.

RESULTS

Characterization of [^{3}H]*chlorambucil uptake*

It has been reported previously that drug uptake over a dose range of 0·002–0·2 mM by both tumour cell strains was rapid, maximum uptake having occurred by 5 min.[1] This result has been confirmed here and by utilizing a technique involving Millipore glass fibre discs (see Methods section), maximal uptake has been shown to have occurred within 30 sec of the addition of chlorambucil to the cell suspension. Under these conditions the uptake of chlorambucil has been shown *not* to proceed against a concentration gradient. Five min after the addition of drug to the cell incubate the concentration of chlorambucil in the cell water is shown to be equivalent to that in the surrounding medium, for both sensitive and resistant cells (see Table 1).

TABLE 1. DATA OBTAINED FROM THE DETERMINATION OF THE INTRACELLULAR CONCENTRATION OF CHLORAMBUCIL AFTER INCUBATION OF YOSHIDA ASCITES SARCOMA CELLS WITH [^{3}H]CHLORAMBUCIL AT 37° FOR 5 min

Cells (10^7)	Wet weight (mg)	Dry weight (mg)	Intracellular water (mg)	Concn of chlorambucil (mM)	
				in the medium	in the cell water
Sensitive	21·1	2·3	13·3	0·20	0·23
Resistant	20·0	1·8	11·0	0·20	0·14

Mean values of six observations.

(i) Effects of various metabolic inhibitors on drug uptake: Equal numbers of sensitive and resistant cells were preincubated for 30 min in the presence of the metabolic inhibitors prior to the addition of chlorambucil. The metabolic inhibitors, dissolved in

PBS and the pH adjusted to 7·4 where necessary, were used at concentrations previously established as inhibitory to the active transport of [^{3}H]lysine into the cell, and in the case of sodium fluoride, inhibitory of the glycolytic processes in these cells.[11] [^{3}H]chlorambucil (0·2 mM) was then added and the cells incubated for 1 hr. As shown in Table 2 the uptake remained unaffected by 0·01–1 mM dinitrophenol (an inhibitor of oxidative phosphorylation); by iodoacetate and sodium fluoride (inhibitors of glycolysis); by ouabain (a specific inhibitor of ATPase).

TABLE 2. EFFECT OF VARIOUS METABOLIC INHIBITORS ON THE UPTAKE OF [^{3}H]-CHLORAMBUCIL BY YOSHIDA ASCITES SARCOMA CELLS AFTER INCUBATION AT 37° FOR 1 hr

Treatment	Conc. (M)	Values expressed as per cent of control	
		Sensitive	Resistant
Sodium fluoride	1×10^{-2}	101	102
Sodium cyanide	1×10^{-4}	104	105
2,4-Dinitrophenol	1×10^{-4}	105	97
	1×10^{-3}	102	101
Sodium iodoacetate	10^{-4}	96	96
	2×10^{-3}	92	100
Ouabain	10^{-4}	100	102
	10^{-5}	102	92
Na-iodoacetate + 2,4-Dinitrophenol	2×10^{-3} 5×10^{-4}	110	100

(ii) Effect of temperature on drug uptake: Uptake of [^{3}H]chlorambucil was found to be independent of temperature in the sensitive cells, since gross accumulation of drug by 5 min was similar at 4° and 37° (Fig. 1). At the lower temperature, in the resistant cells, drug uptake was not inhibited but apparently enhanced (see Fig. 1).

Whilst gross uptake of drug was not inhibited by low temperature, the cellular binding and distribution of the drug was markedly altered. At both 37° and 4°, 5 min after drug addition to the cell suspension, there was a marked accumulation of chlorambucil in the ethanol-soluble extract (see Fig. 2). However unlike the results previously reported after incubation at 37°,[1] there was no subsequent loss of [^{3}H]label from this ethanol extract at 4°, and at this temperature the amount of drug associated with the ethanol-insoluble extract was considerably reduced and showed little increase with time. At 4°, only 5 per cent of the total drug taken up by the cells was bound, in contrast to the observation at 37° where approximately 70 per cent was held in the ethanol insoluble extract after 100 min incubation. The difference in extent of drug binding to sensitive and resistant cells at 4° is not as great as that noted at 37°.

Binding of chlorambucil to DNA, RNA and protein

The sensitive cells accumulated more drug into their nucleic acids and proteins than the resistant cells, and the degree of binding increased with time. The distribution of drug between DNA, RNA and protein isolated from sensitive and resistant cells

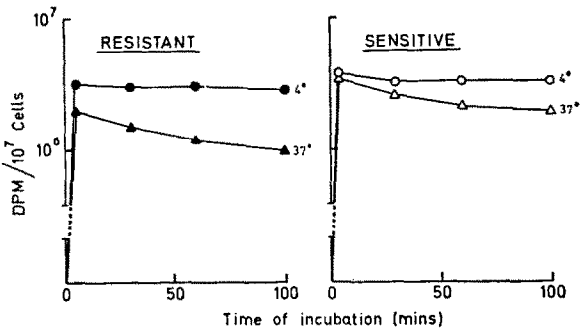

FIG. 1. The effect of temperature on the uptake of [^{3}H]chlorambucil by drug-resistant and drug-sensitive Yoshida ascites cells.

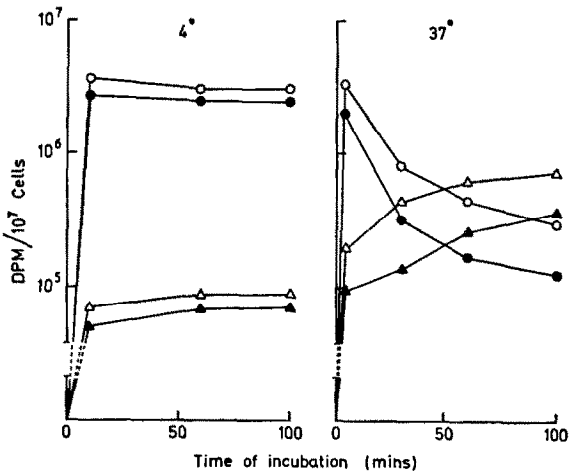

FIG. 2. The effect of temperature on the distribution of [^{3}H]chlorambucil following its uptake of Yoshida ascites cells, into ethanol-soluble and -insoluble extracts.

△——△ sensitive ethanol-soluble extract
▲——▲ resistant ethanol-soluble extract
○——○ sensitive ethanol-insoluble extract
●——● resistant ethanol-insoluble extract.

is shown in Table 3. The extent of binding of chlorambucil in the two cell lines to DNA and RNA is similar. However the initial rate and final extent of DNA labelling in the resistant cells is lower than in the sensitive cells. More drug is bound to protein at all times in sensitive cells, compared with resistant cells. Binding to DNA, RNA and protein is markedly reduced at 4° and the extent of binding does not increase markedly when the period of incubation is prolonged. Furthermore the 2-fold difference in the extent of binding of chlorambucil to the macromolecules of the two cell strains, which was observed at 37°, is much less marked at 4°.

These results indicate that the 2-fold difference in extent of drug accumulation at 37° between the two cell strains may not be solely attributable to the decreased ability of resistant cells to take up the drug, since at 4° it would appear that both cell strains have comparable abilities to accumulate chlorambucil.

TABLE 3. THE DISTRIBUTION OF LABELLED CHLORAMBUCIL BETWEEN DNA, RNA AND PROTEIN (ISOLATED BY A MODIFIED KIRBY PROCEDURE) FROM SENSITIVE AND RESISTANT YOSHIDA ASCITES CELLS FOLLOWING *in vitro* INCUBATION WITH THE DRUG AT A CONCENTRATION OF 0·2 mM AND 10^7 CELLS/ml

	Time of incubation (min)					
	Resistant			Sensitive		
At 37°						
dis./min × 10^5	5	60	100	5	60	100
per mg DNA	0·37*	1·33	1·40	0·62	2·11	2·20
per mg RNA	0·37	1·27	1·73	0·46	1·47	2·07
per mg protein	0·45	2·30	3·20	0·76	4·32	5·71
At 4°						
per mg DNA	0·052	0·077	0·083	0·083	0·140	0·159
per mg RNA	0·083	0·112	0·112	0·181	0·304	0·340
per mg protein	0·257	0·485	0·522	0·410	0·605	0·610

* The DNA isolated from resistant Yoshida ascites cells following 5 min incubation with [^{3}H]-chlorambucil contained 0·37 × 10^5 dis./min/mg DNA. Assuming all the tritium were associated with chlorambucil, this value would be equivalent to approximately 3 × 10^{-8} m-moles chlorambucil per mg DNA.

Metabolism of chlorambucil

It has been previously observed that the resistant cells have a greater ability to modify chlorambucil.[2] Results in Table 4 show that at 4° the ability to modify chlorambucil is completely lost by the sensitive cells, and markedly reduced in resistant cells, since after 100 min incubation 85 per cent of the drug initially accumulated by the resistant cells remains in an unmodified form.

Inactive analogues of chlorambucil, for example, hydrolysed chlorambucil [*N*-bis(dihydroxy-ethyl)phenyl-butyric acid] have been shown to be incorporated less effectively by these cells than the parent compound (see Table 5). This analogue was not degraded by either cell strain at 37°.

TABLE 4. THE EFFECT OF TEMPERATURE ON THE ABILITY OF DRUG-SENSITIVE AND DRUG-RESISTANT STRAINS OF THE YOSHIDA ASCITES CELLS TO MODIFY THE STRUCTURE OF CHLORAMBUCIL (ESTIMATED BY E^1_{258} MEASUREMENTS)

Cells	Temperature	% of total drug initially accumulated by the cells which remains after the following periods of incubation (min)		
		30	60	100
Sensitive	37°	78	73	72
	4°	100	100	100
Resistant	37°	74	54	30
	4°	92	87	85

TABLE 5. A COMPARISON OF THE UPTAKE AND SUBSEQUENT METABOLISM OF CHLORAMBUCIL AND ITS HYDROLYSED DERIVATIVE [*N*-BIS(DIHYDROXYETHYL)PHENYL-BUTYRIC ACID] BY YOSHIDA ASCITES CELLS AT A CONCENTRATION OF 2 mM AT 37°

Cells	Compound	μg compound taken up per 10^8 cells (estimated by E'_{258} measurements) after the following times of incubation	
		5 min	100 min
Resistant	Chlorambucil	280	80
	Dihydroxychlorambucil	118	119
Sensitive	Chlorambucil	290	190
	Dihydroxychlorambucil	125	125

DISCUSSION

Experimental evidence is presented which shows that chlorambucil enters the Yoshida ascites tumour cells by passive diffusion: the drug is not accumulated against a concentration gradient; the rate and extent of drug uptake is not decreased by low temperatures, or by inhibitors of oxidative phosphorylation, or by ouabain. These observations are in contrast to those of Goldenberg *et al.*[12] using HN2, who demonstrated that drug transport into sensitive and resistant L5178Y lymphoma cells was carrier-mediated, and was an active process. The transport of the 3,3-bis(2-chloroethyl) derivative of 5-diazoimidazole)4-carboxamide was also shown to be a temperature-sensitive process.[13] Therefore it would appear that varied mechanisms exist for the transport of alkylating agents, which may account in part for their selective biochemical effects on tumour cells[4,5,14] and their differing therapeutic usefulness in the clinic.[14–16]

The previously reported 2-fold difference in gross uptake of chlorambucil by drug-resistant and drug-sensitive cells at 37° was not apparent at a temperature of 4°; at this temperature both cell strains accumulated comparable quantities of drug. These results therefore indicate that the reduced ability of resistant cells to take up chlorambucil is *not* due to impairment of the transport mechanism, but may be associated with their enhanced ability to metabolize the drug,[2] (which does not occur at 4°).

The impaired uptake of drug at 37° by resistant cells resulted in less extensive binding of ^3H to DNA, RNA and protein. These results are in agreement with previous findings using HN2.[12] The extent of drug-binding to the cellular macromolecular components is considerably reduced at low temperatures and does not increase with time, as is the case at 37°. (The possibility that binding of protein and nucleic acids by chlorambucil may be an enzyme-mediated process is being investigated further.) It has been shown that chlorambucil accumulated in a lipophylic phase (extractable into EtOH) prior to extensive protein and nucleic acid binding at 37°.[1] At the low temperature (4°) the drug also entered this lipophylic compartment, and with the low level of binding at this temperature there was little loss of the drug from the ethanol-soluble extract.

The significance of these uptake characteristics is being investigated further in relation to its relevance to the properties of the drug when administered to tumour bearing hosts.

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