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EFFECTS OF CHLORAMBUCIL TREATMENT ON THE RATES OF RESPIRATION AND GLYCOLYSIS IN DRUG-SENSITIVE AND -RESISTANT STRAINS OF THE YOSHIDA ASCITES SARCOMA

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Abstract—Following *in vivo* administration of a therapeutically effective dose of chlorambucil to rats bearing drug-sensitive and drug-resistant strains of the Yoshida ascites sarcoma, an increased rate of oxygen uptake and aerobic CO_2 production is observed in the sensitive cells. Similar effects are not seen in the drug-resistant cells.

An increased rate of anaerobic lactate production occurs in the sensitive cells 24 hr after *in vivo* treatment. The effect on aerobic lactate production, however, is delayed until 48 hr.

No comparable effects are observed following *in vitro* drug treatment. Inhibition of lactate and CO_2 production and oxygen utilization were achieved only after incubation with drug in a concentration far in excess of an effective *in vivo* dose (assuming uniform distribution throughout the animal). Under these conditions both sensitive and resistant tumour strains were similarly affected.

AN ABNORMALLY high aerobic glycolytic capacity is a well substantiated biochemical characteristic of neoplastic tissue. This observation was first made by Warburg in 1930,[1] but its significance remains controversial.[2,3] The effects of alkylating agents on the glycolytic enzymes of Ehrlich ascites cells *in vitro* have been the subject of considerable study:[4–8] it has been proposed that certain carcinostatic agents exert their effects by decreasing the availability of NAD with a consequent reduction of glycolysis.[7,9]

Information relating to the effects of *in vivo* drug treatment on glycolysis remains inconclusive. No inhibition of anaerobic glycolysis was observed on administering Trenimon to rats carrying the Jensen sarcoma,[10] or following *in vivo* treatment of mice bearing an ascites tumour with TEM.[4] However, Green and Bodansky, expressing their results per milligram nitrogen, report an inhibition of aerobic lactate production in an ascites tumour after treatment with nitrogen mustard, though the lactate formed per cell remained unaltered.[11]

It was of interest to determine whether chemotherapy with chlorambucil would induce glycolytic or respiratory changes in drug-resistant and drug-sensitive strains of a Yoshida sarcoma (ascites). A knowledge of the overall metabolic rate may be relevant to the biochemical effects observed in the tumour strains following chemotherapy, particularly in relation to the transport and metabolism of drugs and of various thiol containing compounds.[12–16]

MATERIALS AND METHODS

Chlorambucil (Leukeran) $(ClCH_2CH_2)_2.N.C_6H_4.(CH_2)_3COOH$ was synthesized in the Chester Beatty Research Institute. Full details of the animal experimentation and tumour transplantation techniques have been given previously.[13, 14]

In vitro *studies.* Ascites cells were harvested from 4–6 day transplants in ice-cold 0·3 per cent phosphate-buffered-saline (PBS) and resuspended in a solution of PBS at 4°. 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.

In vivo *studies.* Chlorambucil was administered subcutaneously in a single dose of 8 mg/kg on the fourth day following tumour transplantation. This dose resulted in complete regression of the sensitive tumour, but was without effect on the resistant cells. Animals were killed by cervical dislocation and the cell suspension prepared as above.

Manometry. Experiments were carried out in a conventional Warburg apparatus at 37°.

The gas phase for aerobic experiments was air, or a N_2–CO_2 (95:5) mixture for anaerobic determinations.

Series 1. The main compartment contained 2×10^7 cells in 2·0 ml of Krebs–Ringer phosphate buffer pH 7·4.[17] 0·3 ml of 0·5 M glucose in phosphate buffer was tipped in from the side arm either 10 or 60 min after equilibration. The centre well contained 0·2 ml of 10 per cent KOH and a strip of filter paper, which was replaced by 0·2 ml of distilled water for determinations of aerobic CO_2 evolution. This was not measured in the presence of a bicarbonate buffer as this was found to lyse the cells. However, the amount of CO_2 absorbed by the phosphate buffer under these conditions was found to be negligible.

Series 2. The main compartment of the flask contained 2×10^7 cells in 2·0 ml Krebs–Ringer phosphate buffer containing 1 per cent glucose: no further addition of glucose was made. The other experimental details were as described for Series 1.

For anaerobic experiments, the buffer was gassed with the N_2–CO_2 mixture for 15 min immediately before use. Flasks containing the cells were gassed for 10 min before initial manometric readings were taken. Similar cell concentrations were used as described for the aerobic experiments, but the centre well remained empty.

Lactic acid determinations. Cell incubates were prepared as detailed above. Samples were taken and added to an equal volume of 0·6 N PCA at 0°, and lactic acid was estimated using a Boehringer Test Combination.

Radioisotope experiments. The experimental conditions were those of Series 2 above. The Warburg flasks were equipped with a gas-tight serum stopper, and cells were incubated for 60 min in the presence of 1-^{14}C-glucose. 0·3 ml of a mixture of ethanolamine and 2-ethoxyethanol (1 : 1) was then injected into the centre well and 1·0 ml of 1N HCl into the main compartment of the flask. Shaking was continued for 1 hr to ensure complete CO_2 absorption. The $^{14}CO_2$ in aliquot of the centre well contents was measured using a Packard Tri-Carb scintillation counter (Model 3375).

RESULTS AND DISCUSSION

Q values were calculated from the experimental data obtained and are expressed in μl of gas per 10^7 cells per hour (10^7 cells = 3 mg dry wt.). The primary experimental

quantities are Q_{O_2} (cell) = rate of oxygen uptake in air; $Q_{CO_2}{}^{air}$ (cell) = rate of CO_2 production in air; $Q_{CO_2}{}^{N_2}$ (cell) = rate of CO_2 production in N_2–CO_2 (95:5).

Effect of glucose on the rates of oxygen uptake and aerobic and anaerobic CO_2 evolution by Yoshida ascites cells

Sensitive and resistant tumour cells had similar Q_{O_2} (cell) and $Q_{CO_2}{}^{N_2}$ (cell) values. Uptake of oxygen was linear with respect to time and no alteration in this rate was observed following the addition of glucose. This absence of a Crabtree effect is in contrast to work previously reported using Ehrlich ascites tumour cells[18, 19] when added glucose caused a reduction in the respiratory rate. This effect was negligible, however, if washed ascites cells were used.[20] Furthermore, isolated Novikoff hepatoma cells showed no Crabtree effect, and this was attributed to synthesis of glycogen following glucose addition.[21] Anaerobic evolution of CO_2 commenced after glucose addition and the rate was also linear over a 60-min period. Therefore in further experiments (Series 2 experimental section) glucose was added either to the cell suspension immediately after equilibration, or was contained in the suspending Krebs–Ringer phosphate buffer.

Effect of in vitro *treatment with chlorambucil*

Varying concentrations of drug were used in order to establish doses at which respiration was (a) inhibited and (b) unaffected. The results are shown in Table 1. At a chlorambucil concentration of 0·15 mM neither oxygen uptake nor CO_2 output were affected in either sensitive or resistant tumour cells. This dose was used previously in studying the uptake of chlorambucil and its effect on the transport of various thiol-containing compounds;[15, 16] it is evident that the differences detected between the two cell strains could not be associated with a direct effect of the drug on respiration of glycolysis. However, a 5-fold increase in drug concentration produced a 50 per cent inhibition of the Q_{O_2} (cell) and $Q_{CO_2}{}^{air}$ (cell) values compared with the solvent-treated control cells of both strains, while a 1·5 mM drug solution caused 70 per cent inhibition. However, the drug was toxic to the cells at much lower doses, and an *in vitro* concentration of chlorambucil of 0·002 mM killed the sensitive cells (i.e. following exposure to the drug the cells were no longer visible on subsequent implantation into healthy rats[22]). Since a concentration 200 times in excess of this dose is required to

TABLE 1. EFFECT OF (*in vitro*) CHLORAMBUCIL TREATMENT ON OXYGEN UPTAKE AND AEROBIC AND ANAEROBIC CO_2 OUTPUT BY YOSHIDA ASCITES TUMOUR CELLS

Tumour cell line	Drug concn (mM)	Q_{O_2}	(cell) S.E.	$Q_{CO_2}{}^{air}$	(cell) S.E.	$Q_{CO_2}{}^{N_2}$	(cell)
Sensitive	zero	12·5	0·3	12·0	0·6	29·0	1·3
	0·15	13·0	0·3	12·9	0·4	31·4	0·5
	0·75	7·9	0·6	6·9	0·8	—	—
	1·5	5·3	0·8	3·5	0·5	—	—
Resistant	zero	12·4	0·5	12·6	0·5	27·7	2·2
	0·15	13·8	0·7	13·7	0·8	30·6	0·6
	0·75	8·8	0·4	7·7	0·6	—	—
	1·5	4·4	0·4	3·6	1·2	—	—

provoke any alteration in the Q values of these cells, it is unlikely that the lethal effects of the drug are mediated through a direct effect on glycolysis or respiration.

Yoshida ascites cells were shown to be susceptible to the action of conventional inhibitors of respiration and glycolysis. For example, sodium fluoride at a final concentration of 0·01 M[18] resulted in a 75 per cent loss of respiratory ability. Chlorambucil had no effect on the action of this inhibitor.

Effect of treatment in vivo *with chlorambucil*

The results of aerobic experiments are shown in Fig. 1. The two cell strains responded differently to *in vivo* treatment with chlorambucil. An increase in the Q_{O_2} (cell) and $Q_{CO_2}^{air}$ (cell) values occurred in the sensitive cells, the rates having doubled 24 hr after

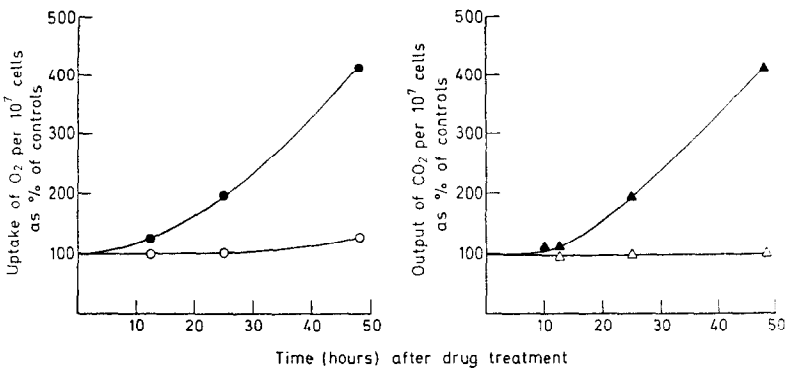

FIG. 1. Effect of treatment *in vivo* with chlorambucil on the oxygen uptake and aerobic CO_2 output by Yoshida ascites cells.

●——● oxygen uptake by sensitive cells.
○——○ oxygen uptake by resistant cells.
▲——▲ CO_2 output by sensitive cells.
△——△ CO_2 output by resistant cells.

(Each point represents the mean of 18 observations. Overall scatter at each point 10 per cent.)

treatment and quadrupled at 48 hr. However, chlorambucil was without effect on the Q_{O_2} (cell) and Q_{CO_2} (cell) values of resistant cells. The increases in Q values in the sensitive cells occurred in parallel with an increase in protein content over the 24 hr following drug treatment. Previous work from this laboratory has indicated that alterations in the levels of glutathione-metabolising enzymes can occur in sensitive cells in response to chlorambucil treatment and it is possible that this drug also modifies selectively the concentration of respiratory and glycolytic enzymes in sensitive cells.[12] However while Green and Bodansky reported increased levels of NAD in Ehrlich ascites cells from HN2-treated animals, they failed to find any significant change in the specific activity of the glycolytic enzymes.[11]

As a result of chlorambucil treatment, the rates of anaerobic CO_2 evolution were unaltered in either strain of ascites cells over a 48-hr period. This suggests that the utilisation of glucose via the Embden–Mayerhoff pathway (and subsequent aerobic CO_2 production), is more susceptible to the action of chlorambucil than the anaerobic utilisation of glucose via the hexose-monophosphate shunt. As confirmation of this

speculation, there was no appreciable alteration in the proportion of $^{14}CO_2$ produced by the sensitive cells from 1-^{14}C-glucose as compared with the total CO_2, therefore indicating that chlorambucil treatment was without effect on the hexose–monophosphate pathway. Other work, on the action of TEM in Ehrlich ascites tumours,[4] also confirmed the greater sensitivity of the respiratory pathway to alkylating agents.

Lactate production following chlorambucil treatment

Untreated tumour cells had high rates of both aerobic and anaerobic lactate production, 220 μg/10^7 cells/hr and 290 μg/10^7 cells/hr respectively. The formation of lactate in the sensitive cells under aerobic or anaerobic conditions was unaffected by exposure to an *in vitro* drug concentration of 0·15 mM. A 1·5 mM concentration of drug was necessary to achieve a significant inhibition (40 per cent in drug-sensitive cells (Table 2). However, *in vivo* drug treatment resulted in a considerable increase in the rate of anaerobic lactate production in sensitive cells 24 hr after administration: no further increase had occurred by 48 hr. Aerobic lactate formation was unaffected at 24 hr, but had increased by 48 hr following drug treatment.

TABLE 2. EFFECT OF CHLORAMBUCIL TREATMENT ON LACTATE PRODUCTION BY DRUG-SENSITIVE CELLS OF YOSHIDA ASCITES SARCOMA

Tumour cell line	Treatment	Drug concn (mM)	Lactate production as percentage of controls μg/10^7 cells/hr*	
			Anaerobic	Aerobic
Sensitive	None	Nil	100	100
	In vitro	0·15	100	100
	In vitro	1·5	60	64
	In vivo	8 mg/kg 24 hr	170	100
		48 hr	168	178

* Mean values of six observations.

These results indicate that *in vivo* administration of chlorambucil increased the glycolytic and respiratory rates in sensitive Yoshida ascites cells, but was without effect on cells of resistant strain: a progressive increase in respiratory activity was recorded over a 48-hr period of study. Under these conditions there appeared to be no increased utilization of the hexose-monophosphate shunt pathway.

Our results also emphasize the different mode of action of chlorambucil under the two sets of experimental conditions, namely treatment *in vivo* and *in vitro*. Effects were only obtained using *in vitro* concentrations of chlorambucil approximately 1000 times greater than those calculated to result from *in vivo* treatment.

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