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Microsomal activation of cyclophosphamide *in vivo*

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IN STUDIES on the metabolism of anti-tumour agents a microsomal system has been developed which is capable of activating Endoxan (cyclophosphamide) *in vitro* to the levels of activation observed with this compound *in vivo*. Activation of Endoxan has previously been reported using liver slices, homogenates and microsomes[1–4] but the degree of activation was not defined.

Methods

Female Wistar rats, 6 weeks old and 220–250 g in weight were used in all experiments. The Walker ascites tumour was routinely passaged by intraperitoneal injection of 2×10^6 cells in saline containing penicillin and streptomycin. Solid tumours were obtained when required by the subcutaneous injection of 2×10^6 ascites cells in the inguinal region.

Rat liver microsomal fractions were prepared essentially by the method described by Grover and Sims.[5] Rat liver was homogenised in 5 volumes of 0·1 M phosphate buffer (pH 7·4) in a Teflon-glass homogenizer and centrifuged at 1000 *g* for 15 min at 5°. The supernatant was collected, spun at 21,000 *g* for 20 min at 2° and the resulting supernatant recentrifuged at 70,000 *g* for 1 hr at 2°. The microsomal pellet was resuspended in approximately the same volume of buffer and centrifuged again at 70,000 *g* for 1 hr at 2°. The successful preparation of microsomes non-toxic to Walker cells *in vitro*, depended on the speed with which the procedure was carried out and on maintenance of low temperature throughout. Preparations were stable for at least 24 hr if deep frozen.

Microsomes from 2·5 g of liver were suspended in 1 ml of saline containing 1·4 mg NADP, 10·4 mg glucose-6-phosphate, 2·1 μl glucose-6-phosphate dehydrogenase, specific activity 140 units/ml (all from Boehringer Corporation (London) Ltd) and 2·1 mg $MgCl_2$ and added to a suspension of tumour cells (about 10^7/ml) in 9 ml of 60% TC199/40% horse serum (Burroughs Wellcome No. 2) together with varying amounts of Endoxan dissolved in 0·1 ml of water. Control incubations consisted of 9 ml of tumour cell suspension mixed with either 1 ml of saline containing microsomes and co-factors or 1 ml of saline only. These mixtures were incubated for 2 hr at 37° with gentle agitation. The viability of the ascites cells in the mixtures was tested by injecting 10^7 cells into groups of five or six animals whose survival times were subsequently observed.

TABLE 1. RELATIONSHIP BETWEEN RAT SURVIVAL TIME AND NUMBER OF WALKER TUMOUR CELLS INJECTED (I.P.)

No. cells	Range of survival times (days)	Mean survival time (days)
10^7	6–8	7·1
10^6	8–11	9·5
10^5	10–13	11·5
10^4	12–15*	13·1

Ten animals per group.
* 30 per cent of the animals did not develop tumours.

Results and discussion

Table 1 shows that for the Walker tumour there is a direct relationship between survival time and the number of cells injected. This table, used in conjunction with the survival times for animals injected with 10^7 incubated tumour cells, makes it possible to estimate the percentages of viable ascites cells present after incubations with Endoxan. Table 2 shows the effect of Endoxan on Walker tumour cells *in vivo*. The dose of Endoxan required to give 90 per cent tumour inhibition compared with untreated tumours is approximately 20 mg/kg which, assuming uniform distribution of the drug throughout the animal, is equivalent to a tumour cell concentration of about 20γ/ml.

TABLE 2. EFFECT OF ENDOXAN ON THE GROWTH OF THE WALKER TUMOUR

Dose Endoxan (mg/kg)	Mean tumour wt. (g)	Inhibition (%)
Controls	48·5	0
1·25	48·0	2
2·50	38·0	22
5·0	40·0	18
10·0	6·5	87
20·0	3·6	93
40·0	0	100
80·0	0	100
200·0	0	100
400·0*	—	—

Endoxan administered intraperitoneally 24 hr after subcutaneous injection of 2×10^6 tumour cells. Tumours dissected out and weighed 12 days later.
* Four out of six died from drug toxicity.

TABLE 3. EFFECT OF INCUBATING ENDOXAN WITH MICROSOMES ON ABILITY TO KILL WALKER TUMOUR CELLS *IN VITRO*

Conditions of *in vitro* treatment of tumour cells prior to injection into rats	Range of survival time of rats (days)	Mean survival time (days)	% Cell kill
Cells only	6–8	7·0	0
Cells + Microsomes + Co-factors	7–9	7·4	0
Cells + Endoxan 800γ/ml	8–9	8·2	56·0
Cells + Endoxan 400γ/ml	7	7·0	0
Cells + Endoxan 200γ/ml	6–8	6·8	0
Cells + Microsomes + Endoxan 80γ/ml*	13–16	14·3	99·95
Cells + Microsomes + Endoxan 40γ/ml†	11–12	11·3	99·10
Cells + Microsomes + Endoxan 20γ/ml	8–13	8·6	76·0
Cells + Microsomes + Endoxan 10γ/ml	7	7·0	0

All incubation mixtures contained Walker ascites tumour cells at a concentration of 10^7 cells/ml. For details of microsome and cofactor concentrations see text.

* Two out of five did not develop tumours.

† One out of five did not develop tumours.

Table 3 shows the activation of Endoxan *in vitro* by the microsomal system. Rats injected with ascites cells that have been incubated for 2 hr at a concentration of 10^7/ml in TCC199 and horse serum in the absence of microsomes and cofactors, have the expected median survival time of 7 days. Similarly, rats injected with ascites cells that have been incubated with microsomes and cofactors have the same survival time, which indicates that these microsomal preparations alone have no toxicity towards these cells. When cells are incubated with Endoxan in the absence of microsomes and co-factors the drug concentration required to cause a 2·5-day extension of rat survival time, which is equivalent to a 90 per cent cell kill (Table 1), is greater than 800γ/ml. However, in the presence of microsomes and co-factors the concentration of Endoxan required to kill 90 per cent of the tumour cells falls to between 20 and 40γ/ml (Table 3).

Microsomal preparations can therefore increase the activity of Endoxan by more than twenty fold. This preparation is being used to investigate the metabolism of Endoxan and to identify the reaction products formed with DNA and with the constituents of sensitive tumour cells that have been incubated *in vitro* with microsomes and Endoxan.

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