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SHORT COMMUNICATIONS

Activation of cyclophosphamide by liver cell cultures

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Cyclophosphamide is a cytotoxic agent used for cancer chemotherapy and more recently as an immunosuppressive agent in the treatment of connective tissue diseases. Studies of the biochemical pharmacology of this compound and the means by which it produces its cytolytic effect on cells, especially the small lymphocyte, have been hampered by the fact that cyclophosphamide is inactive *in vitro* when incubated directly with cells. Cyclophosphamide requires activation to one or more alkylating metabolites by hepatic microsomes [1–3]. Other cells are unable to carry out this conversion or do so to only a very limited extent [4]. Lymphocytes, in our laboratory for example, can be exposed to as much as 1000 μg/ml of cyclophosphamide and remain viable, whereas 1·0 μg/ml of nitrogen mustard will decrease viability by over 90 per cent.

The use of rat embryo liver cells afforded an opportunity to establish a cell culture system that was sensitive to cyclophosphamide and provided a means of investigating the activation and action of this drug on a cellular level.

Rat embryo liver cell lines, E3 and B1, and a control line of rat embryo fibroblasts, BL, were obtained from the laboratory of Dr. Harry Eagle at the Albert Einstein College of Medicine. They were maintained in monolayer culture in Dulbecco–Vogt modified Eagle's medium supplemented with 10% untreated calf serum in 10% CO_2 in room air at 37°. Cyclophosphamide was obtained as Cytoxan® from Mead Johnson Laboratories and prepared freshly by dissolving the contents of a sterile vial in tissue culture medium without calf serum just prior to use.

For studies of direct cytotoxicity, 3–4 × 10^6 E3 cells in 5 ml medium were exposed to 10 μg/ml of cyclophosphamide for 3 hr. The medium containing cyclophosphamide was removed and then the cells were removed by trypsinization with 0·125% trypsin in 0·004 M EDTA and plated on Falcon Microtest II tissue culture plates. Each well received an estimated 500 cells in a volume of 0·2 ml. After 5 days of incubation, the plates were rinsed three times with buffered saline and the viable cells remaining attached in a series of ten wells containing cyclophosphamide-treated and untreated cells were fixed with acetone, stained with giemsa and then counted.

A more sensitive assay for the viability of cells after exposure to cyclophosphamide is that of cloning efficiency, which was performed as follows. Cells were exposed to cyclophosphamide at varying concentrations in plastic petri dishes. The medium containing drug was removed and the cells were treated with trypsin and resuspended in fresh medium without the drug; 5 ml, containing an estimated 100–200 cells, was placed in 25-cm^2 tissue culture flasks. The number of colonies was counted under phase contrast microscopy after 5–7 days of incubation.

Table 1 indicates the direct toxicity of 10 μg/ml of cyclo-

Table 1. Direct toxicity of cyclophosphamide for rat embryo liver cells*

Experiment	Control	Cells per well Cyclophosphamide (10 μg/ml)	Reduction (%)
1	371 ± 16	158 ± 9	57
2	549 ± 19	285 ± 27	48

* Values are expressed as the mean of 10 wells ± S.E.M.

Table 2. Reduction of cloning efficiency by cyclophosphamide (CPA) treatment of rat embryo liver cells

Cell line	Colonies per flask (mean of 3 flasks) Control	CPA (2 μg/ml)	Dexamethasone (10^{-4} M) + CPA (2 μg/ml)*
E-3	209	65	
B-1	144	39	
BL†	145	138	
E-3	71	15	75
E-3	181	46	173

* Dexamethasone for 5 days; CPA for 1 day.
† BL line of rat fibroblasts.

phosphamide for the E3 line of rat embryo liver cells. There was an approximate 50 per cent reduction in the number of viable cells after exposure to the drug.

Using the more sensitive test of measuring cloning efficiency, shown in Table 2, there was a significant loss of viability when the rat embryo liver cell lines E3 and B1 were exposed to 2 μg/ml of cyclophosphamide. The BL line of rat embryo fibroblasts, however, was unaffected by the drug. Pretreatment of the E3 liver cells with the glucocorticoid dexamethasone at a concentration of 10^{-4} M for either 1 or 5 days before challenge with cyclophosphamide protected the cells from the toxic effects of the cyclophosphamide. Whether such treatment altered the activation of cyclophosphamide or protected the cells from the effects of the activation products is not yet established. Analogous experiments with phenobarbital were inconclusive, since incubation of E3 cells with this compound for more than several hours resulted in a variable loss of cloning efficiency in the absence of added cyclophosphamide.

In summary, two lines of rat embryo liver cells were shown to be sensitive to cyclophosphamide *in vitro*. Such culture systems may be useful in studying the pharmacology of this drug at a cellular level.

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REFERENCES

1. J. L. Cohen and J. Y. Jao, *J. Pharmac. exp. Ther.* **174,** 206 (1970).
2. M. Colvin, C. A. Podgett and C. Fenselau, *Cancer Res.* **33,** 915 (1973).
3. T. A. Connors, P. J. Cox, P. B. Farmer, A. B. Foster and M. Jarman, *Biochem. Pharmac.* **23,** 115 (1974).
4. D. L. Hill, W. R. Laster, Jr. and R. F. Struck, *Cancer Res.* **32,** 658 (1972).

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Effects of some epoxides on aryl hydrocarbon hydroxylase activity

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Microsomal monoxygenase and epoxide hydrase are involved in the biotransformation of various toxic and carcinogenic compounds [1–7]. The former enzyme system is generally known to "activate" aromatic compounds into the more toxic or carcinogenic arene oxide (epoxide) derivatives, and the latter enzyme catalyzes the conversion of arene oxides to the less harmful dihydrodiols. The detoxification function of epoxide hydrase has recently been assessed by investigating the effects of some epoxide hydrase inhibitors, such as trichloropropene oxide (TCPO) and cyclohexene oxide, on chemically induced hepatic necrosis or carcinogenesis [8–10]. However, some of the results can not be explained by the inhibitory actions of these oxides on the epoxide hydrase activity. These compounds apparently also affect other enzyme systems. This communication deals with the effects of some epoxide compounds on the aryl hydrocarbon hydroxylase (AHH) activity, one of the catalytic functions manifested by the monoxygenase system.

The epoxides, 1,1,1-trichloropropene 2,3-oxide, 4-chlorophenyl 2,3-epoxypropyl ether and cyclohexene oxide were obtained from Aldrich Chemical Co. These compounds were added to the reaction mixture in 10–20 μl acetone and the solvent had no effect on the reaction. The AHH activity was assayed by measuring the phenolic fluorescent products according to previous procedures [11].

As shown in Fig. 1, TCPO inhibited the liver microsomal AHH activities of both mice and rats. While it inhibited the AHH activity of mouse microsomes at all concentrations, the compound apparently stimulated the AHH activity of rat microsomes at concentrations between 2 and 5 mM.

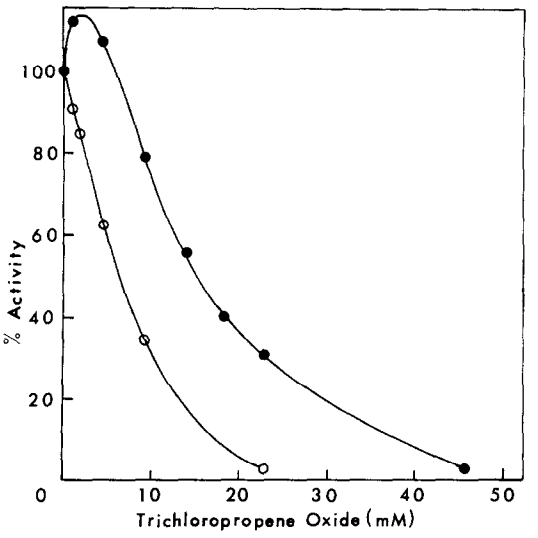

Fig. 1. Inhibition of aryl hydrocarbon hydroxylase by trichloropropene oxide. The assay mixture (1 ml) contained microsomes equivalent to 0·18 mg protein and the incubation time was 10 min. The activities in the absence of an inhibitor were 0·635 mole/min/mg for rat liver microsomes (●——●) and 0·628 nmole/min/mg for mouse liver microsomes (○——○).