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A SIMPLE, SMALL SCALE CYTOTOXICITY TEST, AND ITS USES IN DRUG METABOLISM STUDIES

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Abstract—A small scale method is described, for the estimation of drug toxicity against cells in culture. The usefulness of this test is illustrated by reference to the results of a study of the microsomal activation of cyclophosphamide.

Firstly, it is shown that the microsomal activation of drugs, in this case cyclophosphamide, can easily be demonstrated, *in vitro*, using this technique.

It is also shown that the small scale of the test allows measurements of cytotoxicity to be made on drug metabolites separated by thin layer chromatography, and therefore available in only microgramme quantities. The identification of a number of cytotoxic metabolites of cyclophosphamide is described. An adaptation of the method is described which allows thin layer plates to be "scanned" for cytotoxicity and which has been useful for demonstrating the presence, on the plates, of toxic metabolites of cyclophosphamide which might otherwise have been missed.

The importance of cytotoxicity testing is discussed in relation to the formulation of theories of drug activation in general, and that of cyclophosphamide in particular.

MEASUREMENTS of cytotoxicity play a central role in attempts to find new anti-cancer drugs and to elucidate their mechanisms of action. The most commonly used model system for estimating the effectiveness of drugs against tumour cells is the transplantable animal tumour. Although tissue culture cytotoxicity assays have also been used for this purpose, the culture system is regarded as a less reliable model for human cancer than the animal tumour and therefore less useful for drug screening. However, in studies of drug metabolism and mechanisms of action, the simplicity. flexibility and small scale nature of culture methods gives them considerable advantages over animal tumour systems.

Many methods of assaying cytotoxicity in culture have already been described.[1] In this report a quick, simple, small scale cytotoxicity test system will be described which has been adapted from established techniques for use in a study of the activation of cyclophosphamide, 2-[bis (2-chloroethyl)amino]-tetrahydro-2H-1,3,2-oxazaphosphorine 2-oxide. Cyclophosphamide is an effective anti-cancer agent which has been shown to require activation before exerting its full cytotoxic effect.[2–4] This activation has been shown to occur in liver,[2] and has also been demonstrated using isolated rat liver microsomes.[3–5] The culture methods to be described here have proved to be of considerable value in attempts to separate and identify the active metabolites of cyclophosphamide and it is hoped that the methods will find general application in drug metabolism studies.

EXPERIMENTAL AND RESULTS

Culture of Walker tumour cells. Ascitic fluid was withdrawn from the peritoneal cavity of Wistar rats, bearing a routine passage of the Walker ascites tumour. This fluid was diluted with 10-times its own volume of culture medium (Dulbecco's modified Eagle's medium, supplemented with 10% foetal calf serum, 50 μg/ml streptomycin and 100 i.u./ml of Penicillin). The cells were spun down and resuspended in medium to give a cell concentration of 5×10^5/ml. Five ml aliquots of this suspension were put into 25 cm^2 plastic Falcon flasks which were then gassed with 10% CO_2 in air and incubated at 37·5°.

After 3 days, and then at 4 day intervals, the growing cell suspensions were diluted 1 in 10 in fresh medium. A consistent growth pattern became established after a few weeks and the cells were then considered ready for use in cytotoxicity assays. After 3 months, the cells were discarded and new lines initiated.

Basic cytotoxicity assay (for alkylating agents). Walker cells, from log phase cultures, were resuspended in fresh medium at 2×10^5 cells/ml. This suspension was dispensed in 1 ml amounts, into screw-cap centrifuge bottles. Serial dilutions of the test drug were made up at 100-times the required final concentration and 10 μl of each solution were added to each of a number of bottles containing cells. The bottles were gassed and incubated at 37°, in a shaking waterbath, for 1 hr. After treatment, the cells were washed and resuspended in fresh medium (2 ml per bottle).

Each suspension was dispensed, in 200 μl amounts, into 10 of the wells of a Linbro Microtest plate, incorporating 96, 300 μl, flat-bottomed wells in each 8·5 × 12·5 cm plastic plate. When full, the plates were incubated at 37°, in an atmosphere of 10% CO_2 in air.

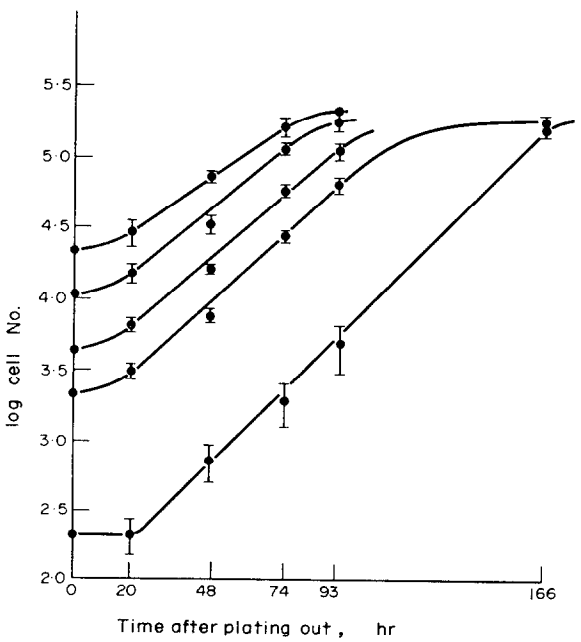

FIG. 1. Growth curves for untreated Walker cells.

Immediately after treatment, and then at 24 hr intervals, two wells from each series of 10 were used for cell counting. The cells, growing in clumps in the wells, were dispersed with a Pasteur pipette and counted in a haemacytometer. Exclusion of 0·2% lissamine green was used as a test for cell viability. Mean cell counts were used to construct growth curves.

Figure 1 shows growth curves for untreated Walker cells growing, in wells, from different initial densities. These curves show that the rate of cell multiplication during the exponential growth phase, and the maximum cell density reached, were both independent of the initial cell density. Each point on the curves in Fig. 1 is the mean of four cell counts on separate wells and it can be seen that the variability between wells of the same series was very small.

The number of cells surviving a particular drug treatment could be estimated by comparing counts of untreated cells with those of treated cells at some time during the exponential growth phase. Counts at 72 hr were used because, earlier than this, dying cells were counted, although they were no longer capable of division. Cell kill could be measured in this way only when drug treatment had not affected the rate of division of the surviving cells. This was checked by comparing the slopes of the growth curves for treated and untreated cells.

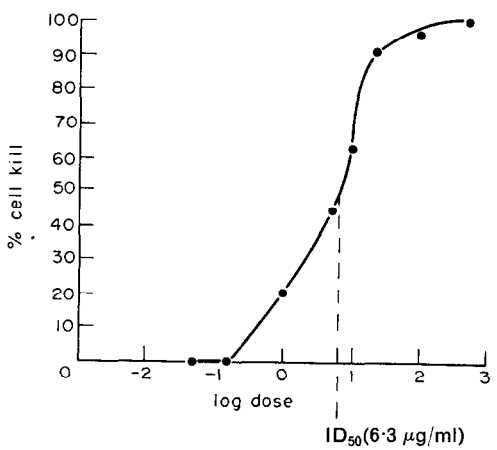

FIG. 2. Dose–reponse curve for cytoxyl alcohol against Walker cells.

Cytotoxicity was expressed as an ID_{50} (50 per cent inhibitory dose), this being the dose which caused a 50 per cent reduction in the increase in cell number over 72 hr of treated as compared with control cells. ID_{50} was read off a dose–response curve of drug dose plotted against cell kill or inhibition of rate of division. One such curve, in this case for the drug cytoxyl alcohol [*N*,*N*-bis (2-chloroethyl)-*N'*-(3-hydroxypropyl)phosphorodiamidic acid], is shown in Fig. 2.

Cytotoxicity of cyclophosphamide, and related compounds. Cyclophosphamide and several related compounds were tested and found to have simple cell-killing effects. The ID_{50}-values for these compounds are given in Table 1.

Activation of cyclophosphamide by rat liver microsomes. Washed rat liver microsomes, prepared as described in the preceding paper, were kindly supplied by Dr. P. J. Cox of this Institute.

TABLE 1. ID_{50} VALUES FOR CYCLOPHOSPHAMIDE AND SOME RELATED COMPOUNDS, TESTED ON WALKER CELLS

Compound	ID_{50} (μg/ml)
Cyclophosphamide (2-[bis(2-chloroethyl)amino]tetrahydro-2H-1,3,2-oxazaphosphorine 2-oxide)	6000
Monochloroethyl cyclophosphamide (2-(2-chloroethylamino)tetrahydro-2H-1,3,2-oxazaphosphorine 2-oxide)	> 3000
4-keto cyclophosphamide (2-[bis(2-chloroethyl)amino]tetrahydro-2H-1,3,2-oxazaphosphorine-4-one oxide)	240
Cytoxyl alcohol (*N,N*-bis(2-chloroethyl)-*N'* (3-hydroxypropyl)-phosphorodiamidic acid)	6·3
Phosphoramide mustard (*N,N*-bis(2-chlorethyl) phosphorodiamidic acid)	0·6
Acrolein	1·0

Cell suspension, containing 4×10^5 cells/ml, was dispensed in 0·5 ml amounts into a number of centrifuge bottles. To each bottle was added 0·25 ml of a solution of "cofactors" containing 9·3 mg/ml glucose 6-phosphate, 1·2 mg/ml $NADP^+$, 5 mg/ml $MgCl_2$, 12·2 mg/ml nicotinamide and 2 units/ml glucose 6-phosphate dehydrogenase, in phosphate buffered saline. The bottles were divided into two groups. Those in one group received 0·25 ml of microsomes (the extract of about 250 mg of rat liver), suspended in phosphate buffered saline, while those in the other group received the same volume of buffered saline.

Serial dilutions of cyclophosphamide were made up in saline, at 100-times the required final concentration. Ten μl of each of these solutions was added to one

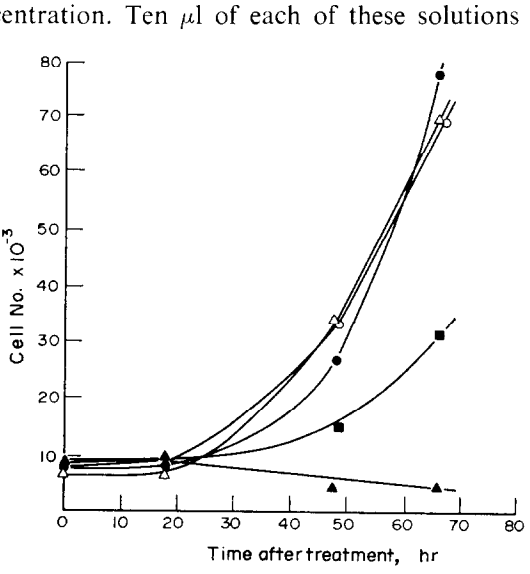

FIG. 3. Effect of microsomes on cyclophosphamide toxicity. Growth of cells treated as follows: (△) 100 μg/ml of cyclophosphamide; (▲) 100 μg/ml of cyclophosphamide plus microsomes; (■) 10 μg/ml of cyclophosphamide plus microsomes; (●) microsomes alone; (○) untreated.

bottle from each of the two groups. The bottles were incubated for 1 hr at 37° and then the cells were washed and plated out in wells as described above.

Figure 3 shows some growth curves obtained in an activation experiment. Microsomes and cofactors had no effect on cell viability and, in the absence of microsomes, cyclophosphamide had no effect at the highest dose used (100 μg/ml). When microsomes, cofactors and cyclophosphamide were present together, 50 per cent kill was obtained with only 10 μg/ml of cyclophosphamide. The ID_{50} of cyclophosphamide alone was found, in other experiments, to be 6000 μg/ml.

Testing of compounds separated by thin layer chromatography. Silica, scraped from a thin layer plate, was found to be toxic to Walker cells in culture. However, if the silica was mixed for 1 min with culture medium and then removed by centrifugation at 3000 *g* for 2 min, the supernatant medium was found to support cell growth perfectly well. Tests showed that, using this method of extraction, more than 90 per cent of a known amount of [^{32}P]cyclophosphamide could be recovered, in the medium, from the silica of a thin layer chromatogram. The [^{32}P]cyclophosphamide recovered in this way was found to be no more toxic than the unlabelled drug, tested in the normal manner.

[^{32}P]cyclophosphamide was incubated with rat liver microsomes and the metabolites were extracted as described in the preceding paper. The metabolite derivatives were separated by thin layer chromatography in chloroform–ethanol (19:1). Four peaks of radioactivity were found.

Silica was scraped from the areas of high radioactivity and from areas between these peaks. The samples were adjusted to 20 mg and extracted with 1 ml of culture medium as described above. 500 μl aliquots of cell suspension, containing 4×10^5 cells/ml were treated with 500 μl of Silica supernatant or supernatant diluted with fresh medium. After a 1 hr treatment, the cells were washed and plated out in wells. Cell kill was estimated from growth curves and used to construct dose–response curves for each of the four peaks. The concentration of each drug metabolite was estimated by measuring the amount of radioactivity in each medium extract.

The ID_{50}-values for extracts from each of the four peaks of radioactivity are given in Table 2. The identification of these four peaks is discussed below, but it should be noted here that the peak at R_f 0·23 was initially identified as cyclophosphamide which had been shown previously to be non-toxic.

Cytotoxicity scanning. The method described above was modified so that thin layer chromatograms could be "scanned" for cytotoxicity. The procedure is shown diagrammatically in Fig. 4. Plates were marked across their width in 0·5 cm strips which

TABLE 2. ID_{50} VALUES FOR WALKER CELLS TREATED FOR 1 hr WITH FOUR METABOLITES OF CYCLOPHOSPHAMIDE, ELUTED FROM THE SILICA OF THIN LAYER CHROMATOGRAMS, DEVELOPED WITH CHLOROFORM–ETHANOL (19:1)

R_f of compound	ID_{50} (μg/ml)
0	3·2
0·11	5·6
0·23	17·0
0·34	1·5

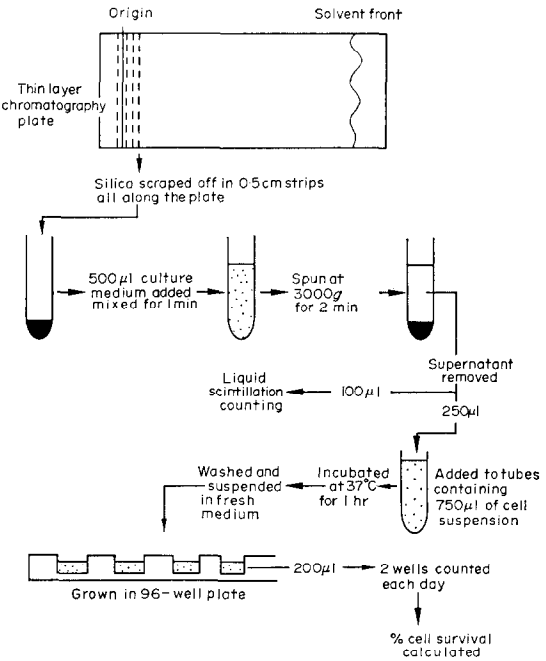

FIG. 4. Scheme for scanning t.l.c. plates for toxicity.

were then scraped individually. The silica samples were put into 1 ml centrifuge tubes, assigned random numbers. Each sample was extracted as before with 500 μl of medium. 100 μl of the supernatant after extraction was used to estimate radioactivity by liquid scintillation counting while 250 μl of each extract was added to 750 μl of cell suspension, containing 2×10^5 cells. The cells were treated for 1 hr, plated out in wells and counted as described above. Percent cell kill was estimated from the growth curves.

Cytotoxicity and radioactivity data were used to construct scans of the chromatograms. Figure 5 shows scans of a chromatogram of the microsomal metabolites of cyclophosphamide, which was developed twice in ether–ethanol (9:1). This solvent system separated four main peaks of radioactivity. The peak around sample 19 (see Fig. 5) was identified as cyclophosphamide which, as expected, was non-toxic. Two peaks were found running faster than cyclophosphamide and extracts from these regions were highly toxic. Material at the origin (samples 1–5) was also found to be toxic and toxicity was found around sample 15, where no peak of radioactivity was seen.

DISCUSSION

Many methods exist for testing drugs on cells in culture (see Ref. 1). The test described here has two advantages over most of these. Firstly, Walker tumour cells are particularly useful for small scale work. These cells have a highly consistent pattern of growth over a wide range of cell density and also grow in suspension, so that no enzyme treatment is required before they can be counted or subcultured.

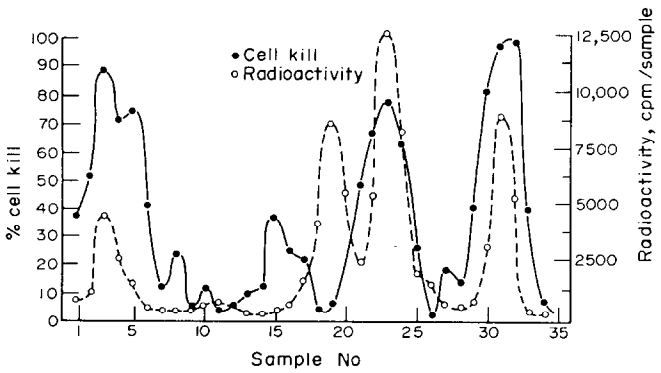

FIG. 5. Cytotoxicity and radioactivity scans of t.l.c. plate used to separate metabolites of [^{32}P] cyclophosphamide.

Furthermore, drug treatment can be carried out on dense cell suspensions, thus allowing many cells to be treated with very small amounts of any compound. Secondly, the use of multi-well culture plates, in which 96 individual cultures of 200 μl capacity can be set up in each small plate, gives a considerable saving in cells, medium and space over the use of tubes, Petri dishes or flasks. For each drug treatment, a series of cultures can be set up and counts can be made at intervals, leaving the remaining cultures undisturbed. Growth curves, constructed from these counts, show clearly the effects of treatment on cell viability and grown rate and also the effects of any random fluctuations in counting accuracy or culture conditions.

The study of cyclophosphamide activation has demonstrated that the small scale, tissue culture, cytotoxicity assay can be useful, in a number of ways, in drug metabolism studies.

Firstly, it was shown that microsomal activation of cyclophosphamide could be demonstrated in culture and that an estimate could be obtained of the lower limit of toxicity to be expected in the active metabolite or metabolites of the drug.

Secondly, the test was useful in attempts to identify these active metabolites. Two approaches to this problem were adopted. Firstly, compounds which had been synthesized because they were thought to be possible metabolites of cyclophosphamide, were tested to see if they were sufficiently toxic to account for the degree of activation observed when cyclophosphamide was incubated with microsomes. Only a small number of compounds were tested but three of these were cytotoxic enough to be possible active metabolites, i.e., cytoxyl alcohol, phosphoramide mustard and acrolein, while monochloroethyl cyclophosphamide and 4-keto cyclophosphamide were relatively non-toxic.

The second approach to identifying active metabolites was to separate the metabolites formed during incubation of [^{32}P]cyclophosphamide with rat liver microsomes. The separation was achieved by thin layer chromatography and, although this gave extremely low yields of each compound, cytotoxicity data could be obtained for each of them. The cytotoxicity test also proved useful as a means of demonstrating the presence of toxic compounds which might otherwise have been missed. When plates were developed in chloroform–ethanol (19:1), four labelled compounds were separated, all of which were toxic. One of these compounds, of R_f 0·34, was identified

as 4-ethoxy cyclophosphamide (see immediately preceding paper by T. A. CONNORS *et al.*) while that of R_f 0·23 appeared to be cyclophosphamide itself. The toxicity of the R_f 0·23 compound, however, suggested that another toxic metabolite was present at the same position as the parent drug. When plates were developed twice with ether–ethanol (9:1), two compounds were separated which had higher R_f-values than the peak identified as cyclophosphamide. These compounds were identified as isomers of 4-ethoxy cyclophosphamide, designated "fast" and "slow" (see preceding paper). Both of these were highly toxic, while the cyclophosphamide peak was completely non-toxic. It appears, therefore, that in the chloroform–ethanol (19:1) solvent system, the slow 4-ethoxy derivative was not separated from cyclophosphamide.

When using isotopically labelled drugs, there is the possibility that certain metabolites might be formed which do not contain the labelled part of the molecule. Such metabolites would not be detected by radiochromatogram scanning but, if cytotoxic, would be detected by cytotoxicity scanning. In the plate scanning experiments described above, a small peak of toxicity was found which did not correspond in position to a peak of radioactivity. This material has, however, not been identified.

The results presented here are consistent with the scheme of activation of cyclophosphamide proposed in the preceding paper.

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