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A NOVEL MIXED HEPATOCYTE-FIBROBLAST CULTURE SYSTEM AND ITS USE AS A TEST FOR METABOLISM-MEDIATED CYTOTOXICITY

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Abstract—The preparation and growth characteristics of a novel mixed hepatocyte-fibroblast culture system is described together with its use as a test for metabolism-mediated cytotoxicity using cyclophosphamide as the compound under test. Cyclophosphamide has a low toxicity *per se* to the fibroblasts but is converted to extremely toxic metabolites when hepatocytes are incorporated with the fibroblasts. This increased toxicity of cyclophosphamide in the presence of hepatocytes can be inhibited by SKF-525A, as can the production by hepatocytes of total alkylating activity from cyclophosphamide. The results are discussed in relation to the relevance of incorporating microsomal suspensions as metabolizing components into the various toxicity tests currently available.

A number of different cell culture systems have been used to assess the cytotoxicity of a wide range of chemicals [1–3]. The criteria of cytotoxicity most commonly employed in these systems include enzyme leakage [4], inhibition of respiration [5], impairment of DNA synthesis [6, 7] and impairment of cell growth [8]. It is now recognized that the majority of chemicals require metabolic activation for their toxic potential to be realized and that this activation is localized predominantly in mammalian hepatocytes [9]. Conversely, a number of chemicals are inactivated by metabolism and this is also preferentially localized in mammalian hepatocytes [9]. Furthermore, it appears that these specialized cellular metabolic functions (viz. normal drug metabolism), which are retained in freshly isolated cells, are either completely absent or not retained in a normal state in culture, even for hepatocytes in culture [10]. What drug metabolizing capability does remain in cultured cells is confined to a very small number of substrates, most of which are polycyclic aromatic hydrocarbons [10]. For this reason the use of most cell culture cytotoxicity tests currently available is restricted to those compounds that are directly cytotoxic and do not require prior metabolic activation.

Attempts have been made to overcome this shortcoming by the introduction of liver microsomes as a metabolizing component into the cell culture system [8, 11, 12]. However, it is highly probable for a number of reasons that this use of microsomes leads to a very abnormal production of toxic metabolites [13]. These reasons include the following: (a) the very high levels of cofactors used which do not represent those existing within intact cells; (b) the lack of active detoxicating enzymes, such as those for conjugation; (c) the possibility that non-microsomal pathways may bring about production of the toxic metabolite; and (d) the probable loss in integrity of the sequence of the various metabolic reactions.

The use of viable, functional adult mammalian hepatocytes, with their full complement of drug metabolizing enzymes, as the metabolizing component is not open to such criticisms and therefore the incorporation of these cells into a suitable culture system would, on these theoretical grounds, be a more rational approach with which to broaden the usefulness of cell culture cytotoxicity tests.

In the course of our studies into the short-term culture of viable rat hepatocytes it became apparent that whilst it was relatively straightforward to isolate appreciable numbers of viable hepatocytes, cultures of these cells for more than 3–4 days was hampered by the rapid overgrowth of fibroblasts. We therefore decided to utilize this novel mixed cell culture system in studies of the metabolism-mediated cytotoxicity of cyclophosphamide (CPA), using the freshly-isolated hepatocytes as the "metabolizing component" and the cultured fibroblasts as the "testing" component.

CPA is an anti-cancer drug that is metabolised in the liver to alkylating agents [14]. The initial step in hepatic metabolism of CPA is microsomal in origin leading to the production of 4-hydroxy CPA which, via its tautomeric aldehyde, can be converted by aldehyde dehydrogenase, a soluble enzyme, to its non-toxic propionic acid, or can undergo a chemical β-elimination to form phosphoramide mustard and acrolein both of which are extremely cytotoxic [8, 15]. The cytotoxicity of CPA is therefore believed to be dependent on the balance between the production of potentially toxic metabolites by microsomal enzymes and the detoxication of these metabolites by soluble enzymes [15]. This example of metabolism-mediated cytotoxicity prompted us to study the usefulness of our novel mixed cell culture system in cytotoxicity tests, using CPA as the nodel compound. The results of this study are presented in this paper.

MATERIALS AND METHODS

Male Wistar/albino rats (60–80 g body weight), bred in the University Animal House and allowed free access to standard laboratory diet and water, were used throughout this study. Tissue culture medium, serum and reagents were obtained from

Gibco-Biocult, Scotland, CPA was purchased from Koch-Light Laboratories, Colnbrook, 4-(*p*-nitrobenzyl)-pyridine (4-NBP) was obtained from Aldrich Chemical Co., Gillingham, and pronase (Type CB) was supplied by Calbiochem, Hereford. 2-diethylaminoethyl-2,2-diphenylvalerate HCl (SKF 525-A) was a generous gift from Smith Kline and French, Welwyn Garden City, Herts, and phosphoramide mustard was a generous gift from Dr. P. J. Cox, Chester Beatty Research Institute, London. "Falcon" tissue culture plasticware was purchased from Scientific Supplies, London.

Hepatocytes were isolated from rat liver by collagenase/hyaluronidase digestion as described previously [16] using sterile techniques throughout and, after assessment of viability by the dye exclusion test [17] (routinely 77–88%), diluted to 2×10^6 viable hepatocytes/ml of culture medium which comprised 10% (v/v) foetal calf serum, 10% (v/v) tryptose phosphate broth in Leibovitz L-15 medium. One ml samples of this cell suspension were pipetted into 10 ml conical flasks and incubated with various concentrations of CPA at 37° in a shaking water bath (approx. 100 oscillations/minute). The CPA and SKF-525A were dissolved in saline at 10 times the desired final concentration and sterilized by membrane filtration prior to use.

At the end of the incubation period the cell suspension was diluted 1 in 10 in complete medium and 1 ml samples of this diluted cell suspension were introduced into plastic tissue culture grade tubes or flasks already containing 2 ml of medium and these were cultured at 37°. Medium was changed 24 hr after initiation of the culture and thereafter at every third day. After 6–8 days in culture, nuclei counts were performed using crystal violet in 0.1 M citric acid [18].

Rat liver fibroblasts essentially free of hepatocytes were obtained as follows. 2×10^6 viable cells freshly isolated from liver were introduced into 8 oz glass bottles and incubated until the fibroblasts formed a confluent monolayer. Cells were then stripped off the glass by means of pronase treatment and these isolated fibroblasts were incubated with CPA and subsequently cultured in Falcon flasks as described above. Light microscopy confirmed that hepatocytes constituted less than 1% of the total cell population both before and after subculturing.

Production of total alkylating activity was assessed as follows. 2×10^6 freshly isolated viable hepatocytes in 1 ml medium were incubated with CPA (final conc. 83 μg/ml) for 1 hr at 37°, in the absence or presence of SKF-525A (final conc. 10^{-5} M). Alkylating activity produced during this time was measured by reaction with 4-NBP essentially according to the method of Sladek [19].

Cultures at various stages of growth were fixed and stained with haematoxylin/eosin, and the stained preparations were photographed using an Olympus CK inverted microscope and a PM 6 camera system.

Statistical analysis was carried out using a standard *t*-test.

RESULTS

Preliminary experiments confirmed that whilst the freshly-isolated cell suspension contained more than 90% hepatocytes, this figure had dropped to less than 1% by day 8 of culture as the fibroblasts overgrew the other cells (Fig. 1). Also, incubation of the cultures with colcemid (0.05 μg/ml for 2 hr) indicated that the hepatocytes in culture were not dividing (mitotic index less than 0.1%). A growth curve (Fig. 1) demonstrated that very little increase in cell number occurred in the first 4 days of culture but that after this time a very rapid increase in cell number took place which started to plateau by the eighth day of culture due to the onset of confluency. This finding was confirmed by light microscopy (Fig. 2) in that the cells predominant after 24 hr in culture were hepatocytes (Fig. 2a); by 4 days in culture the fibroblasts started to grow up (Fig. 2b) such that by the eighth day the fibroblast monolayer was nearly confluent.

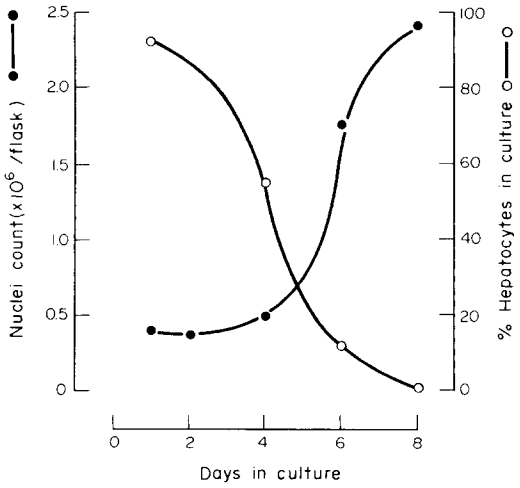
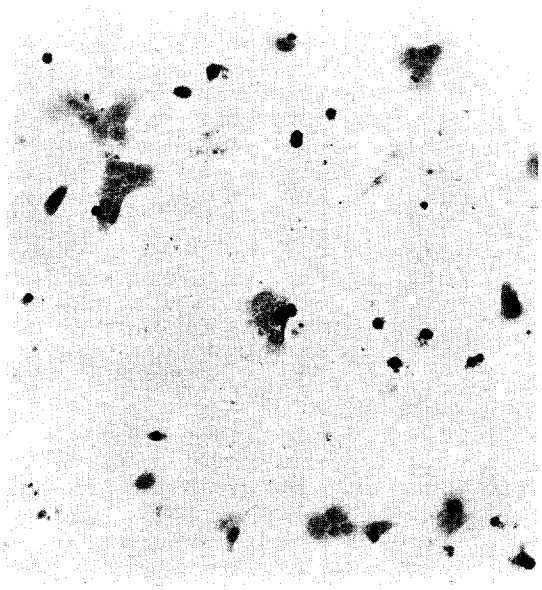
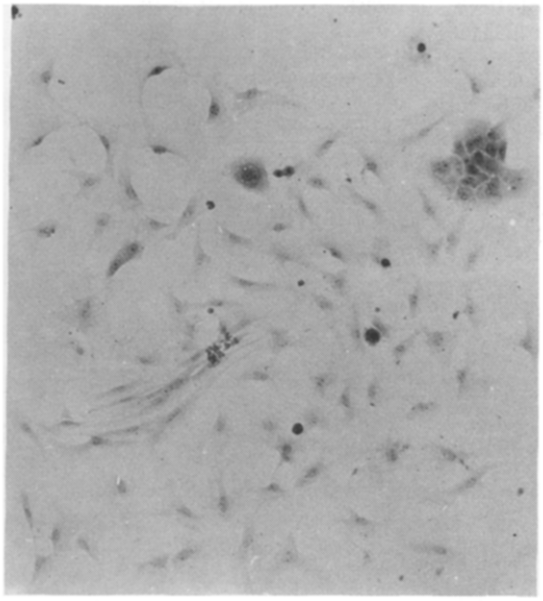


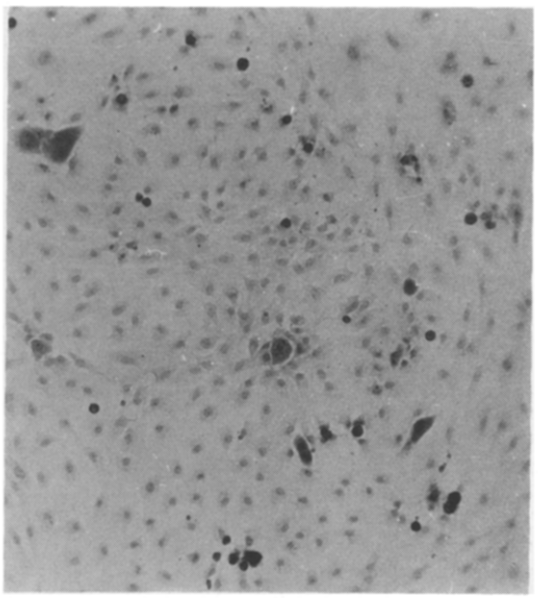
Fig. 1. Growth characteristics of rat liver cells in culture. Cells were inoculated at a level of 2×10^6 cells per 25 cm^2 flask, and nuclei counts and assessment of % hepatocytes performed at the times indicated.



(a)



(b)



(c)

Fig. 2. Light microscopic appearance of rat liver cell cultures at (a) 1 day, (b) 4 day, and (c) 8 days in culture.

When CPA at 91 μg/ml was incubated with freshly-isolated liver cells for varying periods of time and the cells were then cultured, there was a decrease in the number of cells present after 6 days of culture (Fig. 3). The extent of this change increased in a linear manner with increasing incubation times and was not observed when CPA was omitted from the incubation. A very slight (approx. 10%) decrease in cell number was observed after 1 hr incubation of rat liver cells without CPA (Fig. 3) and it is believed this small change is due to binding of some cells to the surface of the glass conical flasks used for the initial incubation, thus making them unavailable for culturing.

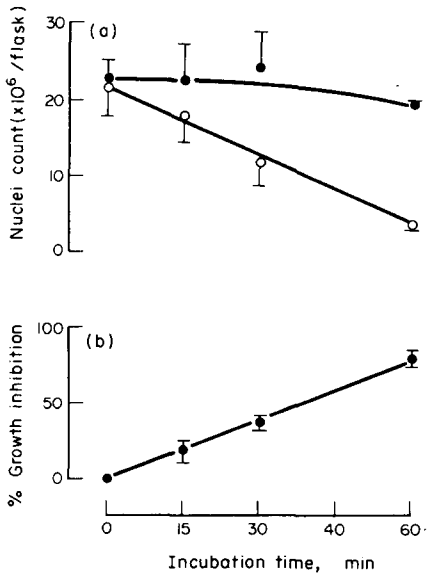


Fig. 3. Effect of incubation time with CPA on the resulting cytotoxicity. (a) Cell suspensions were incubated with (○) and without (●) CPA at a final conc. of 91 μg/ml. The cells were then cultured and counted 6 days later. Each point is mean of 3 experiments with SEM as bar. (b) % Growth inhibition of fibroblasts as a function of incubation time with CPA. Each point is mean of 3 experiments with range as bar.

The effect of varying the CPA concentrations on inhibition of fibroblast growth in the presence and absence of hepatocytes was also studied, and the results are shown in Fig. 4. In the presence of hepatocytes there was a sharp fall-off in the number of fibroblasts recovered after 6 days in culture as the concentration of CPA increased such that at a concentration of 455 μg/ml only 4% of the cell number present in the control cultures was recovered. When the hepatocytes were omitted from the incubation stage no toxicity could be detected even at the highest concentration of CPA used.

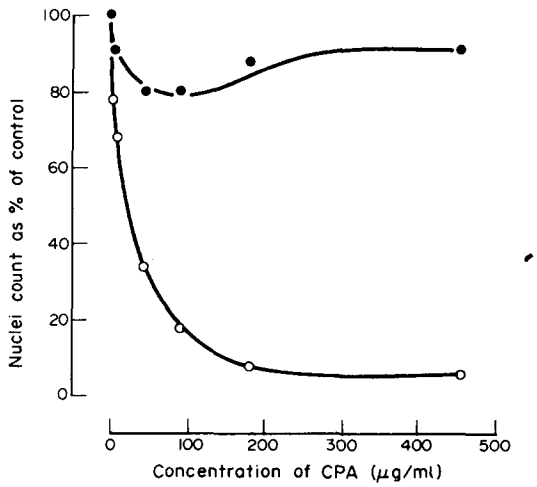


Fig. 4. Influence of CPA concentration on fibroblast growth in the presence (○) and absence (●) of hepatocytes. Cells were incubated for 1 hr, cultured and then counted when confluency was reached.

Table 1. Effect of SKF-525A on the cytotoxicity and alkylating equivalents produced when CPA is incubated with rat liver cells.

Incubation conditions	% Growth inhibition in presence of CPA†	Production of alkylating equivalents*
Control	45.3 ± 5.6 (3)	7.65 ± 0.15 (4)
+ SKF-525A (final conc. 10^{-5} M)	18.7 ± 8.2‡	4.50 ± 0.11‡

Values are mean ± SEM. The number of determinations are in parentheses.
* Expressed as nmoles phosphoramide mustard equivalent produced in 1 h/2 × 10^6 cells. CPA concentration 83 μg/ml.
† CPA concentration 18 μg/ml.
‡ Statistically different from controls at $P < 0.01$.

The results from Figs. 3 and 4 are taken to indicate that CPA is nontoxic *per se* at the levels used in this study but is converted to extremely toxic entities when incubated in the presence of hepatocytes. This contention is substantiated by the findings that co-incubation of the freshly-isolated cells with CPA and SKF-525A, a known inhibitor of microsomal drug metabolism in intact hepatocytes [20], results in a reduction of cytotoxicity which is paralleled by a decrease in the production of total alkylating activity by the cells (Table 1). Initial experiments confirmed that the level of SKF-525A used was not toxic to the cells as judged by growth inhibition. Interestingly, only 3% of the CPA is detectable as alkylating equivalents at the period of maximal cytotoxicity (i.e. 1 hr).

From these results we have been able to construct a dose–response curve for the toxicity of activated CPA (Fig. 5), from which it has been possible to calculate an ID_{50} value (defined as that dose of CPA which inhibits cell growth by 50%) and this value has been compared with other literature values (Table 2).

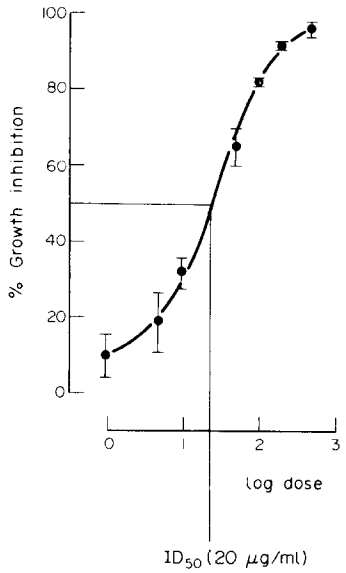


Fig. 5. Dose–response curve for CPA with hepatocytes + fibroblasts. Each point is the mean of 4–6 experiments with SEM as the bar.

DISCUSSION

Previous studies from this laboratory have clearly demonstrated that rat hepatocytes isolated by collagenase/hyaluronidase digestion retain a capability for metabolizing a wide range of xenobiotic chemicals similar to that found in the *in vivo* situation [13, 20]. This conclusion has been further confirmed in this study in that CPA is of low toxicity when incubated with rat liver fibroblasts alone but that this toxicity is considerably enhanced when these fibroblasts are co-incubated with rat hepatocytes as the metabolizing component. The inhibition by SKF-525A both of the cytotoxicity of, and the production of total alkylating activity from, CPA strengthens the argument that metabolism of CPA is required for its full cytotoxicity to be expressed. Attempts to increase the extent of growth inhibition of CPA by the addition of inhibitors of aldehyde dehydrogenase (i.e. disulfiram and *N,N*-bis(dichloroacetyl)-1,8-octane-diamine) proved unsuccessful due to the high toxicity of these compounds. It is interesting to note that at all concentrations tested CPA was not toxic to the hepatocytes themselves as judged by dye exclusion (unpublished work).

Previous work [8, 11, 15] has indicated that activation of CPA by liver microsomes is required for its full cytotoxicity to be expressed. This present study

Table 2. Comparison of ID_{50} values obtained in this study with those reported in the literature.

Control	45.3 ± 5.6 (3)	7.65 ± 0.15 (4)
+ SKF-525A (final conc. 10^{-5} M)	18.7 ± 8.2‡	4.50 ± 0.11‡
KB	>1000	Dolfini *et al.* [11]
KB + mouse liver microsomes	~56 (computed)	
Ascites	6000	Phillips [8]
Ascites + rat liver microsomes	10	
Rat liver fibroblasts	>500	This study
Rat liver fibroblasts + Rat hepatocytes	20	

has extended this argument in that we have demonstrated:

(a) That the activation occurs predominantly in hepatocytes and not to any appreciable extent in other cell types that may be present in liver homogenates. Hepatocyte constitute only 60% of the total cell number of rat liver [21].

(b) That microsomal metabolism is the major cellular pathway for CPA activation. This is based on the fact that the ID_{50} value for CPA in the presence of cells + microsomes (10 μg/ml[8]; see Table 2) is of the same order as that for CPA in the presence of fibroblasts and hepatocytes (20 μg/ml; see Table 2), and that cytotoxicity can be inhibited by SKF-525A (Table 1).

(c) The reactive metabolite(s) of CPA once formed in the hepatocytes can pass readily out from these cells.

In conclusion, we consider that this study has demonstrated the feasibility of using mixed hepatocyte-"tester cell" (i.e. fibroblasts) systems for assessing metabolism-mediated cytotoxicity and that this approach for the testing of liver-generated toxic metabolites which subsequently affect other cell types is probably of more predictive value (to the *in vivo* situation) than is the use of microsome-"tester cell" systems previously used. It is for this reason that the use of isolated viable hepatocytes may also be of value as the metabolizing component in the various microbial mutagenesis [22] and mammalian cell transformation [23] systems currently being employed as tests for chemical carcinogens. The method described in this paper is relatively simple to use as a routine test although it should be borne in mind that this method is probably satisfactory only for those situations in which the reactive metabolite once formed in the hepatocyte is stable enough to be excreted from these cells and taken up by the "tester" cells.

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