Fluorescence-activated sorting of rat hepatocytes based on their mixed function oxidase activities towards diethoxyfluorescein

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The formation of ethoxyfluorescein and fluorescein from diethoxyfluorescein by isolated rat hepatocytes has been used as a basis for separating such cells dependent on their mixed function oxidase activities by fluorescence-activated flow cytometry. Five equal fractions defined by computer-generated regions were isolated. Non-viable cells with low fluorescence (region 1) represented 10–15% of the population, while the remainder with higher mixed function oxidase activities (regions 2–5), were > 95% viable by Trypan Blue exclusion. In region 1, 30% of the viable cells were binucleate, 67% diploid while in region 5, 13% were binucleate and 69% tetraploid. At 3 h after sorting, following attachment to glass coverslips, exposure of cells to methyl methanesulphonate, retrorsine or norethindrone resulted in unscheduled DNA synthesis which was 2-fold higher in the tetraploid-rich region 5, while aflatoxin B₁, benzo[a]pyrene or 2-acetylaminofluorene caused a 5-fold increase in unscheduled DNA synthesis in these cells, relative to the diploid-rich hepatocytes in region 2.

INTRODUCTION

Different areas of the liver lobule can be affected in the acute response to a hepatotoxin which requires metabolic activation by the cytochrome *P*-450-dependent mixed function oxidases. For example, in the male rat, aflatoxin B₁ causes mainly periportal necrosis (Butler, 1964) while with the pyrrolizidine alkaloid retrorsine, the lesion is in the centrilobular area (White *et al.*, 1973). Hepatocytes show both functional and morphological heterogeneity within the lobule (reviewed in Jungermann & Katz, 1982). This heterogeneity applies to the concentrations and species of cytochrome *P*-450 (Gooding *et al.*, 1978; Foster *et al.*, 1986), glutathione concentrations (Smith *et al.*, 1979) and mixed function oxidase activities (Ji *et al.*, 1981).

Isolated hepatocytes have been fractionated using a number of techniques such as sedimentation velocity (Sweeney *et al.*, 1978) and centrifugal elutriation (Sumner *et al.*, 1983; Willson *et al.*, 1985). More recently, liver cells in the periportal or perivenous regions have been selectively destroyed prior to isolation using digitonin perfusion (Lindros & Penttilä, 1985). Miller (1983) used fluorescence-activated flow cytometry to distinguish mouse hepatocytes from an established cell line on the basis of their mixed function oxidase activities.

In this study, fluorescence-activated cell sorting was used to separate and characterize rat hepatocytes on the basis of their mixed function oxidase activities towards

the *O*-de-ethylation of diethoxyfluorescein (I), yielding the fluorescent products, ethoxyfluorescein (II) and fluorescein (III) (Fig. 1). Such hepatocytes were assessed for their abilities to repair damaged DNA and activate model carcinogens. A preliminary account of these studies has been reported (White *et al.*, 1987).

EXPERIMENTAL

Chemicals

Collagenase, human plasma fibronectin, NADPH, glucose 6-phosphate and glucose-6-phosphate dehydrogenase were from Boehringer Mannheim. Fluorescein, methyl methanesulphonate, 2-acetylaminofluorene and benzo[a]pyrene were from Aldrich. Aflatoxin B₁ and retrorsine were gifts from Dr. G. E. Neal and Dr. A. R. Mattocks of this Unit. Ethoxyfluorescein and diethoxyfluorescein were synthesized from fluorescein according to the method described by Miller (1983). The recrystallized products were > 98% pure as judged by h.p.l.c. [*methyl*-³H]Thymidine (specific radioactivity 47 Ci/mmol) came from Amersham International.

Animals

Male Fischer F 344/N rats (140–160 g) were used. These had free access to food and to water. In certain experiments rats were pretreated either with sodium phenobarbitone (0.1%, w/v) in the drinking water for

Fig. 1. Chemical structures of diethoxyfluorescein (I), ethoxyfluorescein (II) and fluorescein (III)

7 days or were given 3-methylcholanthrene (10 mg/kg, 2 mg/ml in triolein) intraperitoneally, once a day for 3 days.

Isolation of hepatocytes: fluorescence-activated cell sorting

Rat hepatocytes were prepared by collagenase perfusion (Paine & Legg, 1978). Viabilities were determined using Trypan Blue exclusion with the aid of a haemocytometer. Preparations with viabilities > 80% were used. Hepatocytes (1×10^7) were suspended in phosphate-buffered saline (10 ml) at 37 °C and incubated in air in a shaking waterbath with diethoxyfluorescein (0.05 mM, added in 50 μ l of dimethyl sulphoxide). After 4 min the suspensions were centrifuged (50 g for 1 min), resuspended in phosphate-buffered saline (10 ml) and used for cell sorting. In some instances for metabolic studies, the cell pellets were resuspended in supplemented Williams medium E [Williams medium E containing foetal calf serum (5%, v/v), gentamycin (0.05 mg/ml), insulin (0.0057 mg/ml) and hydrocortisone sodium succinate (0.048 mg/ml)]. The cells (1×10^7 in 10 ml of medium) were plated out in 15 cm plastic Petri dishes. Plates were incubated at 37 °C in an atmosphere of air/CO₂ (19:1) and harvested at 0.5 h intervals. For metabolite identification in reaction time courses, cells were not centrifuged but a 1 ml aliquot of the reaction mixture was added to ice-cold 1 M-HCl (1 ml). Ethyl acetate extraction and h.p.l.c. were carried out as described below.

Fluorescence-activated cell sorting was carried out using a Cytofluorograf 50H instrument (Ortho Diagnostic Systems, Westwood, MA, U.S.A.). The 488 nm line of an argon laser was used for fluorescence excitation. Emission was measured using a standard Ortho green bandpass filter (no. 3000261002) with 50% T between 509 and 554 nm. Narrow angle forward and 90° scatter were recorded together with fluorescence. Hepatocytes from one incubation mixture were sorted into two regions at a time. Sequential sorts were used to obtain all five regions. Cells (5×10^4) were sorted at a rate of approx. 100/s into plastic multiwell plates containing a 13 mm glass coverslip and 1 ml of supplemented Williams medium E. Before use, the washed sterile coverslips were overlaid for 2 h with 1 ml of Williams medium E containing fibronectin (0.05 mg/ml) to aid cell attachment (Lechner *et al.*, 1985).

At 3 h after sorting, following attachment of the hepatocytes to the coverslips, the medium and non-attached cells were aspirated and replaced with fresh supplemented Williams medium E containing [³H]-thymidine (10 μ Ci) and the test carcinogen dissolved in dimethyl sulphoxide (5 μ l) at the concentrations indicated in the Results section. Hepatocytes were exposed for 18 h after which unscheduled DNA synthesis was assessed using autoradiography as described by Blakey & White (1985). Silver grains over 100 nuclei were counted on each coverslip following 1 week exposure at 4 °C.

Cell ploidy and binucleation in sorted hepatocytes

Cell ploidy was estimated in 2×10^4 nuclei from freshly sorted hepatocytes following ultrasonic disruption of the cells and the addition of ethidium bromide by cytofluorometric analysis as described by Skilleter *et al.* (1983). In one experiment ethidium bromide (7 μ M) was added to intact hepatocytes following incubation with

Fig. 2. Reverse-phase h.p.l.c. separation of diethoxyfluorescein extracted from hepatocyte incubation mixtures

Hepatocytes (1×10^7) were incubated with diethoxyfluorescein (0.05 mM) for 10 min at 37 °C in phosphate buffered saline. Reactions were stopped (1 M-HCl), extracted with ethyl acetate and subjected to h.p.l.c. as described in the Experimental section. Ordinates represent relative absorbance at 242 nm.

diethoxyfluorescein, prior to sorting. Non-viable cells taking up the ethidium bromide and showing red fluorescence (excitation wavelength 488 nm, emission filter 630 nm; Gomperts, 1983), were gated out in the subsequent sorting program.

Estimates of binucleation in sorted hepatocytes after attachment to microscope coverslips as described above were made from phase contrast photomicrographs. Five areas of each coverslip were photographed.

Analysis of diethoxyfluorescein and its metabolites by h.p.l.c.

Reaction time courses stopped with an equal volume of ice-cold 1 M-HCl were extracted three times with ethyl acetate (2 ml). The combined ethyl acetate extracts were concentrated to dryness under N₂ at 37 °C in the dark. Residues made up in methanol (100 μ l) were subjected to reverse phase h.p.l.c. Starting conditions of solvent were methanol/water (1:1, v/v) containing 0.02% (v/v) H₃PO₄. A linear gradient over 10 min (G6, Waters 660 programmer) was to 100% methanol. Flow rate was 1.2 ml/min. The column was a Lichrosorb C-18 (12.4 cm $\times$ 0.4 cm; E. Merck, Darmstadt, Germany). Quantification of metabolites based on absorption monitored at 242 nm was determined from peak areas computed with a Pye-Unicam DP88 integrator. Fluorescence was measured using an excitation wavelength of 242 nm and a high-pass emission filter with 50% T at 418 nm.

Metabolism of diethoxy- and ethoxy-fluorescein by liver microsomal preparations was determined using

Table 1. Microsomal conversion of diethoxyfluorescein to ethoxyfluorescein or ethoxyfluorescein to fluorescein: effect of pretreatment of rats with mixed function oxidase inducers

Reaction mixtures (1 ml) contained washed hepatic microsomal fractions (1–1.3 mg of protein) from control, phenobarbitone- or 3-methylcholanthrene-pretreated rats, a NADPH-generating system and either diethoxyfluorescein or ethoxyfluorescein (0.3 mM). After 4 min at 37 °C, reactions were stopped with ice-cold 1 M-HCl. Ethyl acetate extracts were subjected to h.p.l.c. as described in the Experimental section. Results represent the mean $\pm$ S.E.M. for four experiments.

Pretreatment	Reaction rate (nmol/min per mg of protein)	
	Diethoxyfluorescein to ethoxyfluorescein	Ethoxyfluorescein to fluorescein
None	0.20 $\pm$ 0.01	0.07 $\pm$ 0.01
Phenobarbitone	0.23 $\pm$ 0.02	0.08 $\pm$ 0.01
3-Methylcholanthrene	0.32 $\pm$ 0.01	0.61 $\pm$ 0.05

reaction mixtures (1 ml) containing: potassium phosphate buffer, 0.1 M, pH 7.4; MgCl₂, 2 mM; EDTA, 0.5 mM; glucose-6-phosphate dehydrogenase, 2 units; microsomal fraction, 1–1.3 mg of protein. Reactions were started by the addition of diethoxyfluorescein (5–50 μ M) or ethoxyfluorescein (1–200 μ M) dissolved in dimethyl sulphoxide (5 μ l) and stopped with 1 M-HCl (1 ml). Extraction and quantification of metabolites were carried out as described above. Hepatic microsomal fractions were prepared as described previously (White, 1980). Microsomal protein was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as standard. Estimations of K_m values were computed using the procedures described by Wilkinson (1961).

RESULTS

Metabolism of diethoxyfluorescein

Fig. 2 shows the h.p.l.c. separation of diethoxyfluorescein and its major Phase I metabolites, ethoxyfluorescein and fluorescein, after extraction from hepatocyte incubation mixtures. The rates of formation of both metabolites from diethoxyfluorescein were linear with time up to 15 min: for ethoxyfluorescein 0.39 ± 0.07 and for fluorescein 0.11 ± 0.01 nmol/min per 10^6 cells (mean $\pm$ S.E. for four experiments). Recoveries of authentic ethoxyfluorescein and fluorescein (1 nmol) added to such mixtures were $97 \pm 4\%$ and $98 \pm 1\%$ respectively. The use of dimethyl sulphoxide as a solvent for the substrate up to a concentration of 0.5% (v/v) did not have any significant effect on the rate of the reactions.

Using liver microsomal suspensions, the apparent K_m values for the *O*-de-ethylation of diethoxyfluorescein to ethoxyfluorescein was 25 ± 5 μ M while that for ethoxyfluorescein to fluorescein was 97 ± 13 μ M (mean $\pm$ S.E. for four experiments). In order to obtain some data on the species of cytochrome *P*-450 involved in these reactions, the effects of pretreating rats with the microsomal mixed

Fig. 3. Fluorescence-activated analysis of hepatocytes incubated with diethoxyfluorescein: dependence on time of incubation

Hepatocytes (1×10^7) were incubated in phosphate-buffered saline (10 ml) at 37 °C in air for the times indicated with diethoxyfluorescein (0.05 mM). Following centrifugation and resuspension cells were subjected to fluorescence-activated analysis using excitation wavelength 488 nm; emission filter > 530 nm. The zero time point was obtained prior to the addition of diethoxyfluorescein. Ordinates represent relative number of cells.

function oxidase inducers, phenobarbitone or 3-methylcholanthrene, were investigated. Table 1 shows that the conversion of ethoxyfluorescein to fluorescein was induced about 8-fold by pretreatment with 3-methylcholanthrene, while the inductive effect of this compound on the de-ethylation of diethoxyfluorescein was much less marked.

Fluorescence-activated sorting of hepatocytes following incubation with diethoxyfluorescein

When hepatocytes were incubated with the non-fluorescent diethoxyfluorescein, they fluoresced green when examined by u.v. fluorescence microscopy. Fluorescence-activated analysis of such cells show the presence of two sub-populations. One of these showed fluorescence which increased with the time of incubation, and the other smaller sub-population (about 10% of the total) showed a low fluorescence which did not increase with incubation time (Fig. 3). An incubation time of 4 min was chosen for the remaining experiments. The population defined at this time (Fig. 4a) was sequentially sorted into five fractions defined by equal computer-generated regions (1–5). The initial viability of cells from region 1 immediately after sorting as shown by Trypan Blue exclusion was $< 5\%$ while for cells in the remaining regions, viabilities were $> 95\%$. In all experiments, the percentage of cells in region 1 corresponded well with the percentage of non-viable cells assessed by Trypan Blue exclusion prior to sorting. The elimination of non-viable hepatocytes which take up ethidium bromide and show

Fig. 4. Fluorescence-activated sorting of hepatocytes following incubation with diethoxyfluorescein

(a) Fluorescence-activated analysis of hepatocytes. Conditions were the same as described in the legend to Fig. 3 except an incubation time of 4 min was used. The population was divided into five equal computer-generated regions as shown on the basis of the fluorescent intensity of individual hepatocytes. Region 1 has cells with the lowest, and region 5 the highest, fluorescence. (b) Appearance of hepatocytes 4 h after sorting. Hepatocytes were sorted into multiwell plates containing supplemented Williams medium E (1 ml) and allowed to attach to fibronectin coated glass coverslips for 3 h. After changing the medium, cells were photographed using phase-contrast (magnification $\times 115$).

Table 2. Distribution of binucleate cells and nuclear ploidy in sorted hepatocytes

Fluorescent-activated sorting of hepatocytes into five equal regions was carried out following 4 min incubations with diethoxyfluorescein. Cells in region 1 had the lowest, region 5 the highest, fluorescence. The number of binucleate cells was determined from phase-contrast photomicrographs: 400 cells were counted following a 3 h attachment period to fibronectin-coated glass coverslips. Ploidy was determined in 2×10^4 nuclei by fluorescent-activated analysis (excitation 488 nm, emission 630 nm) following ultrasonic disruption of cells and treatment with ribonuclease and ethidium bromide. Results represent the mean $\pm$ S.E. for four experiments.

Region	Binucleate cells (%)	Nuclear ploidy (%)		
		Diploid	Tetraploid	Octaploid
Unsorted	18 $\pm$ 1	37 $\pm$ 3	55 $\pm$ 4	3.0 $\pm$ 0.4
1	30 $\pm$ 6	67 $\pm$ 6	31 $\pm$ 4	0.1 $\pm$ 0.03
2	26 $\pm$ 3	54 $\pm$ 9	39 $\pm$ 8	0.8 $\pm$ 0.7
3	24 $\pm$ 3	35 $\pm$ 4	59 $\pm$ 3	1.0 $\pm$ 0.6
4	19 $\pm$ 1	31 $\pm$ 4	64 $\pm$ 4	1.2 $\pm$ 0.1
5	13 $\pm$ 1	24 $\pm$ 2	69 $\pm$ 4	2.8 $\pm$ 0.3

red fluorescence resulted in the loss of the sub-population in region 1. The appearance of a representative sample from each region, 3 h after plating onto glass coverslips, is shown in Fig. 4(b). The plating efficiency of cells with

the highest mixed function oxidase activities towards diethoxyfluorescein (regions 4 and 5) consistently appeared higher than those in the remaining regions (Fig. 4b), although this effect was not quantified.

Fig. 5. Time courses for the loss of diethoxyfluorescein from hepatocytes

Hepatocytes (1×10^7) were incubated for 4 min at 37 °C with diethoxyfluorescein (500 nmol) in phosphate-buffered saline (10 ml). Following centrifugation and resuspension, cells were transferred to plastic Petri dishes and incubated at 37 °C. At 0.5 h intervals cells were harvested, centrifuged and the concentration of diethoxyfluorescein and its metabolites in the cell pellets were estimated using reverse phase h.p.l.c. as described in the Experimental section. Δ , Diethoxyfluorescein; $\circ$, ethoxyfluorescein; $\square$, fluorescein.

Binucleation and ploidy of sorted hepatocytes

Table 2 shows the percentage of binucleated cells in the different sorted regions. Those cells with the lowest mixed function oxidase activities towards diethoxyfluorescein (regions 1 and 2) had the highest proportion of binucleate cells. Table 2 also shows there was a gradation in nuclear ploidy, viable cells in regions 1 and 2 having the highest proportions of diploid, and region 5, tetraploid, cells. If non-viable cells are excluded from region 1, the proportion of diploid hepatocytes is increased to $84.1 \pm 1\%$.

DNA repair and metabolic activation of model carcinogens

Fig. 5 shows the time course for the loss of diethoxyfluorescein from hepatocytes following an initial 4 min incubation period. At 3 h after centrifugation and plating out, < 6% of the original concentration of diethoxyfluorescein remains and only low levels of the major Phase I metabolites, ethoxyfluorescein and fluorescein, are present in the cells. This time was therefore chosen for subsequent studies on the metabolic activation of model carcinogens. Barth & Schwarz (1982) showed that fluorescein after intracellular enzymic generation is secreted into the intercellular clefts. It cannot be excluded in the present study that some loss of fluorescence from the hepatocytes with time is due to conjugation reactions. It seems probable that these more water-soluble derivatives like fluorescein itself would be eliminated from the cells and would not interfere with activation of DNA repair.

Table 3 shows the extent of unscheduled DNA synthesis as assessed by autoradiography. Because of their low viabilities, region 1 cells were excluded from this study. Exposure of the sorted hepatocytes to the direct alkylating agent methyl methanesulphonate resulted in 2-fold higher levels of unscheduled DNA synthesis in the predominantly tetraploid cells (region 5) than in the mainly diploid cells (region 2). The remaining chemicals examined require metabolic activation in order to exert their genotoxic effects. Following exposure to retrorsine or norethindrone, unscheduled DNA synthesis was also enhanced 2-fold in the region-5 hepatocytes compared with those of region 2. A 5-fold difference between these regions was observed after dosing with 2-acetylaminofluorene, aflatoxin B₁ or benzo[a]pyrene.

DISCUSSION

Fluorescence-activated cell sorting based on mixed function oxidase activities requires a non-fluorescent substrate which is metabolized to products which are fluorescent at neutral pH values and are retained intracellularly. Diethoxyfluorescein fits these requirements. Other commonly used substrates used for the fluorescence assay of these enzymes, e.g. ethoxycoumarin or ethoxylresorufin, are not particularly satisfactory. The

Table 3. Unscheduled DNA synthesis in sorted hepatocytes

Hepatocytes were sorted into five regions by fluorescence-activated analysis as described in the legend to Fig. 3. After allowing the cells a 3 h attachment period, the supplemented Williams medium E was replaced with one containing [³H]thymidine and the carcinogens at the doses indicated. After 18 h, the coverslips were washed, fixed and dipped in photographic emulsion. Samples were exposed for 1 week at 4 °C. The number of silver grains over 100 nuclei and the corresponding area of cytoplasm was counted for each experiment. Results represent the mean $\pm$ s.e. for two experiments.

Compound	Concn. (M)	Regions ...	Unscheduled DNA synthesis [(grains/nucleus) – background]				
			2	3	4	5	Unsorted
Acetylaminofluorene	1×10^{-5}		6 $\pm$ 1	11 $\pm$ 2	12 $\pm$ 2	29 $\pm$ 2	19 $\pm$ 2
Aflatoxin B ₁	1×10^{-8}		14 $\pm$ 2	19 $\pm$ 2	22 $\pm$ 2	62 $\pm$ 3	19 $\pm$ 4
Benzo[a]pyrene	1×10^{-5}		12 $\pm$ 1	26 $\pm$ 2	36 $\pm$ 2	63 $\pm$ 3	25 $\pm$ 2
Retrorsine	1×10^{-4}		7 $\pm$ 1	10 $\pm$ 1	15 $\pm$ 1	19 $\pm$ 2	12 $\pm$ 1
Norethindrone	1×10^{-4}		6 $\pm$ 1	7 $\pm$ 2	8 $\pm$ 1	11 $\pm$ 1	7 $\pm$ 1
Methyl methanesulphonate	1×10^{-5}		6 $\pm$ 1	7 $\pm$ 1	13 $\pm$ 1	11 $\pm$ 1	14 $\pm$ 1

maximum fluorescence of coumarin is only obtained at alkaline pH values (Rogiers *et al.*, 1986), while resorufin is readily reduced by DT-diaphorases (Nims *et al.*, 1984). Conversion of diethoxyfluorescein to ethoxyfluorescein is poorly catalysed by mouse hepatocytes (Miller, 1983). The present study using rat hepatic microsomal systems shows that the mixed function oxidases responsible for the metabolism of diethoxyfluorescein to ethoxyfluorescein are not phenobarbitone-inducible and are only slightly induced by pretreatment of rats with 3-methylcholanthrene (Table 1). The extent of induction by 3-methylcholanthrene is very much less than the > 50-fold increases found using ethoxyresorufin as substrate (Burke & Mayer, 1983), suggesting different forms of cytochrome *P*-450 may be involved.

In addition to the range of mixed function oxidase activities, the sorted hepatocytes exhibit a gradation of binucleate cells and degree of nuclear ploidy (Table 2). In the predominantly tetraploid cells (region 5), twice as much DNA repair occurs as a result of exposure to the direct alkylating agent methyl methanesulphonate as in the diploid hepatocytes (region 2, Table 3). It is not known if this is simply due to tetraploid nuclei having twice the DNA content or to a greater expression of the enzymes involved in DNA repair.

It is impossible from the present data to attribute the sorted regions to locations within the liver lobule. Hepatocytes with the highest mixed function oxidase activities are generally associated with the centrilobular area (Ji *et al.*, 1981; Jungermann & Katz, 1982). The degree of nuclear ploidy has also been claimed to increase from the periportal to centrilobular region (Guguen-Guillouzo & Guillouzo, 1986). Exposure of sorted cells either to retrorsine, which results in hepatic centrilobular necrosis (White *et al.*, 1973), or to aflatoxin B₁, which causes periportal necrosis (Butler, 1964), effected higher levels of unscheduled DNA synthesis in hepatocytes from region 5 relative to those from region 2 (Table 3).

Present results suggest that aflatoxin B₁, acetylaminofluorene and benzo[*a*]pyrene are activated to a greater extent in tetraploid hepatocytes than in diploid ones. This conclusion is supported by the findings of Neal *et al.* (1976) and Schwarze *et al.* (1984) that the administration of either aflatoxin B₁ or 2-acetylaminofluorene to rats in their diet causes a selective destruction of the higher ploidy hepatocytes, leaving a predominantly diploid cell population.

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