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Use of aggregating cell cultures for toxicological studies

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Summary. Relatively simple techniques are now available which allow the preparation of large quantities of highly reproducible aggregate cultures from fetal rat brain or liver cells, and to grow them in a chemically defined medium. Since these cultures exhibit extensive histotypic cellular reorganization and maturation, they offer unique possibilities for developmental studies. Therefore, the purpose of the present study was to investigate the usefulness of these cultures in developmental toxicology. Aggregating brain cell cultures were exposed at different developmental stages to model drugs (i.e., antimitotic, neurotoxic, and teratogenic agents) and assayed for their responsiveness by measuring a set of biochemical parameters (i.e., total protein and DNA content, cell type-specific enzyme activities) which permit a monitoring of cellular growth and maturation. It was found that each test compound elicited a distinct, dose-dependent response pattern, which may ultimately serve to screen and classify toxic drugs by using mechanistic criteria. In addition, it could be shown that aggregating liver cell cultures are capable of toxic drug activation, and that they can be used in co-culture with brain cell aggregates, providing a potential model for complementary toxicological and metabolic studies.

Key words. Aggregating cell cultures; brain cell cultures; liver cell cultures; teratogenesis; toxicology; antimitotic drugs; cholera toxin.

Introduction

The technique of rotation-mediated aggregating cell culture has been introduced by Moscona[26, 27], who showed that freshly isolated immature cells of any fetal organ are able to reassociate spontaneously in vitro, giving rise to three-dimensional, organotypic cultures. Subsequently, this culture system has been applied mainly for developmental studies of the brain. These investigations showed on morphological as well as on biochemical grounds that aggregating fetal brain cells are able to mimic several morphogenetic events occurring in vivo (e.g., cell migration, synaptogenesis, myelination), and that they finally attain a high degree of cellular organization and differentiation in vitro [10, 26, 31]. Several methodological improvements, notably the replacement of enzymatic tissue dissociation by a simple mechanical sieving procedure [17] and the introduction of a chemically defined culture medium [15], greatly simplified this culture technique, and at the same time increased the yield and the reproducibility of the cultures for multidisciplinary investigations, for example combining morphology, biochemistry, and molecular biology. Aggregating brain cell cultures can thus be grown routinely in batches of up to 150 replicate cultures, each containing several thousand aggregates. For statistical evaluations it was found sufficient to assay triplicate or quadruplicate cultures. Thus, with one culture batch up to 50 different culture conditions can be evaluated simultaneously (e.g., the dose-response relationships of up to a dozen different compounds) by assaying for a series of biochemical parameters.

The wide range of developmental processes exhibited by aggregating brain cell cultures offers additional advantages for toxicological investigations. The study of certain teratogens may be restricted to the period of cell proliferation and early differentiation (occurring during the first week of culture), whereas cell-specific toxicity (e.g., neurotoxicity, toxic effect on astrocytes or oligodendrocytes, demyelinating effects) may appear at a more advanced developmental stage. Aggregating cell cultures offer the possibility to study drug action at successive developmental stages on identical cultures.

Since in the organism many xenobiotics are subject to hepatic metabolism or activation, an attempt was made to extend the technique of aggregating cell culture to liver cells. A method was developed for the preparation and maintenance of fetal rat liver cells in a chemically defined medium, and it could be demonstrated that these cultures are able to express and maintain liver-specific functions comparable to perinatal rat liver in vivo [9]. Thus, it is now possible to prepare separate aggregating cell cultures from liver and brain of the same rat fetuses, and to use these cultures either separately or in co-culture at different developmental stages for toxicological investigations.

Materials and methods

Materials. Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose, but no pyruvate, and basal medium Eagle (BME) vitamin mixture were purchased from GIBCO. Human transferrin, bovine insulin, 3,3'5-triiodothyronine, hydrocortisone-21-phosphate, 1-β-D-arabinofuranosylcytosine (Ara-C), gentamicin sulfate, glutamic acid (potassium salt), lipoic acid, linoleic acid, acetylated trypsin (type V-S), trypsin inhibitor, methotrexate, diazepam, diethylstilbestrol, phenobarbital (sodium salt), and diphenylhydantoin were obtained from Sigma. Cyclophosphamide was from Koch-Light. Phosphoenolpyruvate (sodium salt), ATP (sodium salt), pyruvate kinase (from rabbit muscle), and acetyl coenzyme A were purchased from Boehringer, Mannheim. Epidermal growth factor (EGF; 'receptor grade') was obtained from Collaborative Research, Waltham, MA, USA. Cholera toxin and L-α,β-diaminopropionic acid were from Calbiochem. Prior to use, cholera toxin was dialyzed against 10^3 volumes of HEPES buffer (1 mM) pH 7.4, containing 0.9% (w/v) NaCl. [methyl-^{3}H]Thymidine (25 Ci/mmol), [5-^{3}H]thymidine (27 Ci/mmol), and [methyl-^{14}C]thymidine (61 mCi/mmol) were obtained from Amersham.

Acetyl-coenzyme A[1-^{14}C] (45.1 mCi/mmol), acetylcholine iodide [acetyl-^{14}C] (4.7 mCi/mmol), and L-[1-^{14}C]glutamic acid (46 mCi/mmol) were purchased from New England Nuclear. Prior to use, the L-[1-^{14}C]glutamic acid was purified by passage over a Dowex AG 1×8 (Cl^-) column [29].

Aqueous radioactive samples were mixed with Ready-Solv EP scintillation cocktail (Beckman) and counted in a Beckman LS 9000 liquid scintillation spectrometer.

Cell culture. For culture preparation, tissues were taken from Wistar rat fetuses on the 15th day of gestation. Time-pregnant rats were obtained from Madörin AG, Füllinsdorf, Switzerland.

Aggregate cultures of either the telencephalon or the whole brain were prepared from mechanically dissociated cells as described previously in detail [10, 12, 13, 15, 17].

Aggregate cultures of the liver were prepared from cells isolated from fetal liver by a modification of the sequential digestion technique of Chessbeuf et al.[4], as described in detail by Guigoz et al.[9].

Aggregate cultures were kept under constant gyratory agitation at 37 °C, in an atmosphere of 10% CO_2 and 90% humidified air. The serum-free DMEM was supplemented with transferrin (1 µg/ml), insulin (800 nM), triiodothyronine (30 nM), hydrocortisone-21-phosphate (20 nM), BME vitamins, vitamin B_{12}, lipoic acid, linoleic adic, retinol, α-tocopherol, L-carnitine, and trace elements [10, 13, 15]. Liver cell cultures received on day 3 a mixture of fatty acids bound to bovine serum albumin (instead of free linoleic acid), L-proline (11.5 mg/l), and EGF (10 µg/ml) as described by Guigoz et al.[9].

Analytical procedures. For biochemical analyses, the aggregates of each flask were washed three times with 5 ml of ice-cold phosphate-buffered saline and homogenized in 0.4 ml of 1 mM potassium phosphate buffer, pH 6.8, using glass-glass homogenizers. The homogenates were briefly sonicated, divided into aliquots for the different assays, and stored at −80 °C.

The activity of choline acetyltransferase (ChAT; EC 2, 3, 1.6) was determined by a modification [40] of the Schrier and Shuster [35] method, and corrected for the portion of non-specific activity, determined by omission of choline in the assay mixture.

Acetylcholinesterase (AChE; EC 3.1.1.7) activity was measured by a modification [40] of the method of Reed et al.[30] and Ehrenpreis et al.[6], and corrected for the portion of pseudocholinesterase (EC 3.1.1.8) activity[17].

Glutamic and decarboxylase (GAD; EC 4.1.1.15) was determined by a modification[40] of the Wingo and Awapara method[41].

Glutamine synthetase (GS; EC 6.3.1.2) activity was assayed by a modification[28] of the method of Pishak and Phillips[29]. L-[1-^{14}C]glutamic acid was used as precursor, and phosphoenolpyruvate/pyruvate kinase was used as ATP-regenerating system[28].

The activity of 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP; EC 3.1.4.37) was measured according to the method of Sogin[36].

Total DNA was measured by the fluorometric method of Downs and Wilfinger[5]. A preparation from herring sperm (Boehringer) was used as a standard.

Protein was determined by the Folin phenol method[22], using bovine serum albumin (Serva) as a standard.

All values presented unter 'Results' are the means of three to four different cultures, allowing to maintain SEM < 5%. Based on our experience using the Student's t-test, values may be considered as significantly different (in a first approximation) when they deviate by >15%. (For a detailed statistical evaluation of some of our data, see Marazzi et al., this review.)

Results and discussion

1. Drugs affecting proliferating cells. Previously, it has been shown[10] that in aggregating cell cultures of fetal rat telencephalon, the mitotic activity is restricted to the first two weeks in vitro. During this phase, almost all glial cells and some neurons complete their last rounds of cell divisions before entering the prolonged period of cell differentiation. Since drugs affecting mitotically active cells have a high teratogenic potential, cytosine arabinoside (Ara-C) has been taken as a model substance in order to study the effects of antimitotic drugs at different developmental stages of brain cells. Previous experiments have shown that Ara-C causes a dose-dependent reduction of DNA synthesis in aggregating cell cultures of fetal rat telencephalon[8]. A single dose of Ara-C (0.4 μM) added on day 3 was sufficient to block almost completely the DNA synthesis on day 7. Therefore, in the present study, such cultures were treated with Ara-C (0.4 μM) at three different developmental stages, corresponding to the periods of early (days 3 to 5) and late (days 6 to 8) mitotic activity, as well as an early postmitotic period (days 14 to 16). All cultures were harvested on day 21 and assayed for their content of protein and DNA, as well as for their levels of enzyme activities specific for neurons (choline acetyltransferase, ChAT; acetylcholinesterase, AChE; glutamic acid decarboxylase, GAD), astrocytes (glutamine synthetase, GS) and oligodendrocytes (2'3'-cyclic nucleotide 3'-phosphodiesterase, CNP), respectively.

The results (table 1) show that Ara-C treatment during the early phase of mitotic activity caused an almost total loss of glial enzyme activities, a considerable decrease of AChE (−38%) and GAD (−27%) activity, as well as a reduction of total DNA (−47%) and protein (−40%), whereas the total ChAT activity per culture remained unchanged. Ara-C treatment during the late phase of mitotic activity still greatly affected the glial enzyme activities (−74% of GS activity; −96% of CNP activity) and reduced total DNA (−39%) and protein (−21%), but did not decrease (in some cases even increased) the neuronal enzyme activities. Ara-C treatment during the early postmitotic phase had little (CNP activity, DNA) or no effect on the various parameters examined. These results suggest that Ara-C (0.4 μM) was specifically cytotoxic for proliferating cells, and thus affected the final cellular composition of the cultures as a function of the mitotic activity of the different cell types at the time of Ara-C treatment. Therefore, it can be assumed that during the first 5 days in culture, almost all glial cells were proliferating, whereas only certain types of neurons (e.g., GABAergic but not cholinergic neurons) were mitotically active. Oligodendrocytes thus showed the longest period of proliferation. These general conclusions were supported by the nearly identical results obtained by treating

Table 1. Selective cytotoxic effect of cytosine arabinoside, ^{3}H-thymidine, and diaminopropionic acid on proliferating cells in aggregating cell cultures of fetal rat telencephalon

Treatment	Period of treatment (day in vitro)	Protein, DNA and enzymatic activities per culture, day 21 (% of untreated controls[4])						
		Prot	DNA	ChAT	AChE	GAD	GS	CNP
Ara-C[1]	3–5	60	53	98	62	73	10	3
	6–8	79	61	129	100	117	26	4
	14–16	94	88	104	99	97	96	88
^{3}H-Thd[2]	3–5	58	55	99	72	75	5	0
	6–8	75	63	149	109	140	16	3
	14–16	97	86	120	107	114	85	77
L-DAP[3]	3–5	61	53	107	76	84	26	11
	6–8	69	56	153	101	115	27	28
	14–16	106	98	104	121	99	103	113

[1] Cytosine arabinoside (Ara-C): 0.4 μM; [2] [^{3}H-methyl]-Thymidine (^{3}H-Thd): 2.0 μCi/ml (spec. act. 25 Ci/mmol);[3] L-α,β-Diaminopropionic acid (L-DAP): 0.1 mM; [4] The values of the untreated controls (mean of 4 cultures ± SEM) were: Protein (Prot): 6.73 ± 0.18 mg/flask, DNA: 586 ± 30 μg/flask, Choline acetyltransferase (ChAT): 650 ± 32 pmol/min/flask, Acetylcholinesterase (AChE): 134 ± 42 nmol/min/flask, Glutamic and decarboxylase (GAD): 5.9 ± 0.2 nmol/min/flask, Glutamine synthetase (GS): 863 ± 43 nmol/min/flask, 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP): 11.9 ± 0.5 μmol/min/flask.

the cultures with ^{3}H-thymidine (2 μCi/ml), as shown in table 1. As found for Ara-C, the effects of ^{3}H-thymidine were strictly dose-dependent (not shown). Since [^{3}H-methyl]thymidine and [5-^{3}H]thymidine caused identical effects, whereas [^{14}C-methyl]thymidine was totally ineffective (data not shown), it can be concluded that ^{3}H-thymidine incorporated into the DNA of dividing cells was lethal to these cells due to the local ionizing radiation.

A dose-dependent arrest of the mitotic activity in aggregating cell cultures of fetal rat telencephalon comparable to that observed with Ara-C treatments was obtained also with L-α,β-diaminopropionic acid (L-DAP; data not shown). The pattern of responses observed by treating cultures with 0.1 mM L-DAP (table 1) is very similar to those found with Ara-C and ^{3}H-thymidine treatments, respectively. Therefore, it can be concluded that all of these three agents are able to eliminate selectively the mitotically active cells, albeit through different mechanisms of action.

In order to use a more general model for routine teratogenicity/toxicity testing, aggregate cultures were prepared from the entire fetal brain, instead of the telencephalon only. However, due to the more advanced developmental stage of certain brain areas as compared to the telencephalon[21], cultures of whole brain cells showed considerably less mitotic activity. Nevertheless, besides the known antimitotic drugs (e.g., cyclophosphamide, see Table 5; 5-fluorouracil and hydroxyurea, data not shown), several putative teratogens (e.g., methotrexate, diazepam, diethylsilbestrol) revealed a more pronounced (but not exclusive) toxicity for proliferating cells (Table 2).

Conceivably, there may exist teratogenic compounds which stimulate, rather than inhibit or eliminate, mitotically active cells. Phorbol ester tumor promoters may serve as examples of drugs belonging to this category. It has been shown that phorbol 12-myristate 13-acetate (PMA) and mezerein greatly enhance the differentiation of astrocytes, while progressively reducing the mitotic activity in aggregating brain cell cultures[11]. This effect was found to be specific for proliferating astroblasts in a three-dimensional cellular environment.

2. *Neurotoxic drugs.* Aggregating cell cultures of fetal rat brain contain a great variety of neuronal cell types, reflecting closely the cellular composition of the original brain region used for culture preparation[10, 25]. In these cultures, the fetal neurons are able to reorganize and to differentiate extensively, as evidenced in mature cultures by the presence of morphologically mature synapses[15, 20, 23, 34, 38], the high specific activities of neurotransmitter synthesizing enzymes[10, 31], biosynthesis, storage and release of neurotransmitters[17, 33], and the occurrence of spontaneous bioelectrical activity[37]. Therefore, this culture system seems to offer a convenient model to study neurotoxic compounds.

Cholera toxin has been chosen as a model substance to study possible effects of neurotoxic drugs in aggregating brain cell cultures. Thus, aggregating cell cultures of fetal rat telencephalon were treated at different developmental stages with cholera toxin (10^{-7} M) and assayed on day 34 for the different neuronal and glial enzyme activities. As shown in table 3, treatment of these cultures on day 4 greatly reduced both the neuronal and glial parameters as well as total DNA and protein content. Cultures treated on day 7 showed an almost total absence of the neuronal enzyme activities, whereas the levels of glial enzyme activities (GS, CNP) per culture were reduced to about half of untreated controls. Since the specific activities of GS and CNP were even somewhat higher than in control cultures, these results suggest that cholera toxin has a general toxic effect on all immature cells, whereas it shows a specifically neurotoxic action in more differentiated cultures. Indeed, treatment of more advanced cultures (on days 13 and 20, respectively) still greatly reduced the neuronal parameters, but did not affect the glial enzyme levels (table 3).

Aggregating brain cell cultures have also been used to study the selective neurotoxicity of kainic acid[17], organophosphorous compounds[39], and choline mustard analogues[3]. In the screening experiments of putative teratogens, some compounds (e.g., phenobarbital, diphenylhydantoin) showed a predominant effect on neuronal parameters (table 4).

3. *Drugs affecting myelination.* Aggregating cell cultures have the advantage that they cover a wide range of devel-

Table 2. Examples of drugs affecting preferentially the proliferating cells in aggregating cell cultures prepared from fetal rat brain

Compound	Dose[1] (μg/ml)	Protein, DNA and enzymatic activities per culture, day 12 (% of untreated controls)						
		Prot	DNA	ChAT	AChE	GAD	GS	CNP
Methotrexate	0.05	65	60	72	89	42	14	16
	0.50	67	59	72	72	40	11	13
	5.00	60	37	50	66	34	10	12
Diazepam	1	73	89	76	78	73	61	74
	10	70	81	63	53	41	30	30
	100	22	40	2	5	0	1	0
Diethylstilbestrol	1	112	104	102	103	96	99	114
	10	114	109	90	90	91	100	117
	100	50	31	50	38	21	19	5

[1] Period of treatment: day 3 to day 11.

Table 3. Maturation-dependent, neuron-specific action of cholera toxin in aggregating cell cultures of fetal rat telencephalon

Day of treatment	Protein, DNA and enzymatic activities per culture, assayed on day 34 Prot (mg)	DNA (µg)	ChAT (pmol/min)	AChE (nmol/min)	GAD (nmol/min)	GS (nmol/min)	CNP (µmol/min
– (untreated)	9.9 ± 0.3	373 ± 8	2543 ± 35	301 ± 11	12.7 ± 0.6	1145 ± 42	8.9 ± 0.4
4	1.5 ± 0.1	87 ± 1	63 ± 23	3.5 ± 1.3	0.31 ± 0.05	158 ± 13	0.68 ± 0.07
7	2.5 ± 0.1	120 ± 1	0	1.8 ± 0.1	0.16 ± 0.01	513 ± 10	4.3 ± 0.09
13	4.7 ± 0.3	212 ± 7	125 ± 29	22 ± 4	0.97 ± 0.18	1254 ± 59	8.5 ± 0.2
20	5.4 ± 0.4	274 ± 4	362 ± 34	39 ± 3	1.26 ± 0.06	1310 ± 62	10.2 ± 0.3

[1] Cultures were treated for 24 h with cholera toxin (10^{-7} M).

Table 4. Examples of drugs with a preferential action on neurons in aggregating cell cultures prepared from fetal rat brain

Compound	Dose[1] (µg/ml)	Protein, DNA and enzymatic activities per culture, day 12 (% of untreated controls) Prot	DNA	ChAT	AChE	GAD	GS	CNP
Phenobarbital	1	94	92	82	91	85	98	97
	10	92	88	85	89	75	95	95
	100	89	95	66	79	55	93	104
Diphenylhydantoin	20	102	104	74	73	58	89	100
	40	85	91	55	53	34	71	79
	80	56	60	44	36	23	37	25

[1] Period of treatment: day 3 to day 11.

opmental events. This also allows the study of processes such as myelination, which occur relatively late in development. Therefore, this culture system has been used to examine the influence of hormones[1, 16] and growth factors[2] on myelin synthesis. Furthermore, it has been shown that in aggregating brain cell cultures, demyelination can be induced by low calcium or hexachlorophen[24], demonstrating the usefulness of this system to study drug-induced demyelination.

4. *Biotransformation of drugs.* In the organism in vivo, drugs are often metabolically transformed and thereby either detoxified or, in some cases, activated. The liver is known to play a major role in the metabolism of toxic compounds. Therefore, an attempt was made to combine a possible system for toxicity screening with a second that would provide the metabolizing capacity of the liver, by using co-cultures of aggregates from brain and liver. To this end, aggregating cell cultures were prepared individually from the brains and livers of the same rat fetuses, and grown separately in serum-free medium for the first 8 days. Liver cell cultures were pretreated on day 6 with phenobarbital (3.2 mM) in order to activate drug metabolizing enzymes. Cyclophosphamide, a compound known to require metabolic activation for its teratogenic effect, was added on day 8, either to brain cell aggregates alone or to co-cultures of liver and brain cell aggregates. On day 12, all cultures were assayed for the different neuronal and glial enzyme activities. The results (table 5) show that cyclophosphamide i) affected predominantly proliferating brain cells, and ii) was more potent in co-cultures of liver and brain cell aggregates than in cultures of brain cell aggregates alone. Although more experiments are needed to fully evaluate this co-culture system for toxicological studies, the present results suggest that it can serve to study drugs subject to hepatic activation.

Conclusions

Aggregating cell cultures prepared from fetal brain span a wide range of ontogenetic processes occurring in the normal brain, and therefore offer the possibility to perform toxicological studies at different developmental stages as well as in highly differentiated, functional cultures. For routine tests in developmental toxicity, a set of parameters may be chosen, representing different metabolic processes, general developmental events, as well as specific characteristics of a given cell type. Clearly, the usefulness of this system depends on the careful selection of convenient screening assays. Therefore, more work is

Table 5. Increased cytotoxicity of cyclophosphamide in fetal rat brain cell aggregates co-cultured with fetal rat liver cell aggregates

Culture[1]	Cyclophosphamide[2] (µg/ml)	Protein, DNA and enzymatic activities per culture, day 12 (% of untreated controls) Prot	DNA	ChAT	AChE	GAD	GS	CNP
B	300	90	84	114	82	80	81	78
B	500	80	77	127	86	85	71	60
B + L	300	65	53	101	53	38	23	17
B + L	500	56	45	47	31	13	10	11

[1] B: aggregate cultures of fetal rat brain cells, L: aggregate cultures of fetal rat liver cells pretreated on day 6 with phenobarbital (3.2 mM); [2] Period of treatment: day 8 to day 11.

needed in order to develop and apply powerful, multidisciplinary diagnostic criteria.

The results of the present study indicate that each class of teratogens may produce a characteristic pattern of effects which is dose-dependent, highly reproducible, and discernible from unspecific toxic effects observed at higher drug concentrations. Furthermore, it was found that the concentrations of teratogens effective in aggregating brain cell cultures were, in general, comparable to the teratogenic concentrations determined in chick (Kucera and Burnand, this issue) and rat (Schmid and Cicurel, this issue) embryo cultures (see also Marazzi et al., this issue).

Aggregate cultures prepared from fetal liver cells can be used as a complementary system for studies of drug metabolism. Toxic compounds requiring hepatic activation can be examined in co-cultures of liver and brain cell aggregates. In addition, each system may be used separately to determine the relative potencies of analogues of a given class of toxic substances, and to study the mechanisms of action of teratogenic or otherwise toxic substances.

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The use of primary cultures of adult rat hepatocytes to study induction of enzymes and DNA synthesis: Effect of nafenopin and electroporation

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Summary. Primary cultures of adult rat hepatocytes maintained in a well-differentiated state, in a chemically defined medium containing 2% DMSO, have been utilized to study the effect of non-mutagenic hepatocarcinogens such as the peroxisome proliferator nafenopin. The parameters chosen in this in vitro system were those that paralleled the major in vivo effects of nafenopin on the liver, mainly: the proliferation of the endoplasmic reticulum and induction of cytochrome P-452, the proliferation of the peroxisome compartment and the induction of cyanide-insensitive β-oxidation of fatty acids and the stimulation of liver growth as measured by the DNA synthetic activity of the hepatocytes.
In this review, we also describe the morphology of hepatocyte cultures prepared from previously electroporated hepatocytes and the potential for the use of electroporation to introduce growth related genes into hepatocyte cells to study the mechanisms of hepatocyte growth at the molecular level. In addition we describe the formation of endoplasmic reticulum whorls in these cultures as a consequence of nafenopin treatment. 'Whorl formation' by hepatotrophic chemicals has been previously shown to occur in vivo; in this report, it is described for the first time in vitro.
Key words. Hepatocyte cell cultures; cytochrome P-450; peroxisome proliferation; DNA synthesis; nafenopin; electroporated hepatocytes; DMSO.

Introduction

Considerable efforts are currently being exerted to reduce the use of animals for toxicity testing. Consequently, primary cultures of adult rat hepatocytes, as an alternative to the whole animal, are now widely employed for assessment of genotoxic potential and cytotoxicity mechanisms[4, 12]. Other parameters of interest to us and others are those concerning the hepatocarcinogenic or tumor promotive action of chemicals which were negative in standard tests of genotoxicity[16]. Many of these chemicals induce liver growth accompanied by increases in specific enzyme activities. For example, the long-term administration of the hypolipidemic agent nafenopin and other peroxisomal proliferators has been found to cause liver tumors[15–17] accompanied by liver growth, proliferation of the endoplasmic reticulum and the peroxisomal compartment[7, 18, 25], induction of microsomal cytochrome P-452 and the peroxisomal cyanide insensitive β-oxidation system[1, 14]. The relationship between such effects and tumor development is not clearly understood, although a correlation between peroxisome proliferation and hepatocarcinogenesis has been suggested[15, 16]. Since the target organ is the liver, hepatocyte cultures provide an excellent in vitro system to assess the effects of nafenopin and similar chemicals on the above-mentioned parameters and to study the cellular mechanisms of these toxic manifestations with the minimal use of animals.
However, the use of hepatocyte cultures for such studies has been hindered by the short life-span of the cells in culture (3–4 days), the loss of differentiated functions and the rapid and continual decline in their cytochrome P-450 content under a variety of culture conditions[21, 22]. A major advancement in hepatocyte culture techniques was the introduction of serum-free chemically defined media, designed specifically for the maintenance of liver cells in culture. With such systems it is possible to per-