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PHASE I AND PHASE II XENOBIOTIC BIOTRANSFORMATION IN CULTURES AND CO-CULTURES OF ADULT RAT HEPATOCYTES

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Abstract—The aim of this study was to measure the activity of phase I and II key enzymes in the biotransformation of xenobiotics and their inducibility by phenobarbital (2 mM) in two currently used *in vitro* models, namely adult rat hepatocytes, conventionally cultured or co-cultured with rat epithelial cells derived from primitive biliary duct cells. For phase I, the cytochrome P450 content and the enzymic activities of 7-ethoxycoumarin *O*-deethylase and aldrin epoxidase have been determined, for phase II glutathione *S*-transferase activity was measured. In conventional cultures, all phase I parameters investigated declined continuously as a function of culture time. Two mM phenobarbital had inducing effects on 7-ethoxycoumarin *O*-deethylase and glutathione *S*-transferases but not on aldrin epoxidase. In co-cultures, after an initial decrease, a steady state situation developed for all the parameters measured, lasting for at least 10 days. The cytochrome P450 content, the 7-ethoxycoumarin *O*-deethylase, aldrin epoxidase and glutathione *S*-transferase activities were maintained from 3 to 4 days on at 25, 100, 15 and 50%, respectively, of their corresponding value obtained for freshly isolated hepatocytes. After phenobarbital treatment, the parameters mentioned were significantly increased with the exception of the aldrin epoxidase activity of which the inducibility was nearly completely lost.

During the last few years alternative *in vitro* models for pharmacotoxicological studies of xenobiotics have gained popularity, not only because of the increasing criticism on *in vivo* experiments by animal-welfare groups but also because of improved technology and the existence of a number of economic, scientific and ethical advantages proper to these *in vitro* techniques [1].

It is generally accepted that freshly isolated hepatocytes, kept in suspension for some hours, represent a suitable model for biotransformation, acute toxicity and mechanistic studies of xenobiotics [2–4].

For the investigation of long-term effects, primary monolayer cultures of hepatocytes have often been proposed. However, these cultures undergo a number of irreversible biochemical and morphological changes making the results obtained not necessarily comparable with the *in vivo* situation [3, 5–9].

A co-culture model of adult rat hepatocytes with rat epithelial cells, probably derived from primitive biliary duct cells, has been proposed by the Guguen-Guillouzo group [10, 11]. From their work it appears that the life time of the primary cultures is extended and that the biochemical and morphological integrities are better kept in function of culture time [10, 11]. It seems that the cytochrome P450 content could be maintained, although the isoenzyme pattern and its variation in function of culture time have not yet been described.

If an *in vitro* system is to be used for pharmacotoxicological studies, it is important that the content and the activity of cytochrome P450 is preserved and that it remains inducible. Furthermore phase I metabolites must be further detoxified by phase II conjugation. Therefore, the aim of this study was to measure some important key enzymes in the biotransformation of xenobiotics and their inducibility by phenobarbital (PB‡) in co-cultures, kept under the same conditions as reported in the literature [10]. These results were compared with those obtained for conventional cultures.

For phase I biotransformation, the cytochrome P450 content and the enzymic activities of 7-ethoxycoumarin *O*-deethylase (ECOD) and aldrin epoxidase (AE) have been determined. Both are dependent on different cytochrome P450 isoenzymes [12–16].

Concerning phase II, glutathione *S*-transferase (GST) activity and its isoenzyme pattern were analysed. The results of the latter have been published separately [17–19].

MATERIALS AND METHODS

Chemicals. Crude collagenase type I, bovine serum albumin fraction V, bovine insulin, β-glucuronidase type H-1 and 1-chloro-2,4-dinitrobenzene were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.) Aldrin, dieldrin and heptachlorepoxide were from Riedel-De Haën (Hannover, F.R.G.) and 7-ethoxy- and 7-hydroxycoumarin from Aldrich (Brussels, Belgium). All media, fetal calf serum and trypsin-EDTA solution were purchased from Gibco (Brussels, Belgium)

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‡ Abbreviations: ECOD, 7-ethoxycoumarin *O*-deethylase; AE, aldrin epoxidase; GST, glutathione *S*-transferase; CDNB, 1-chloro-2,4-dinitrobenzene; PB, phenobarbital.

and hydrocortisone hemisuccinate from Roussel (Paris, France). All other compounds were readily available commercial products and were used without further purification.

Liver samples. Rat livers were obtained from outbred male Wistar albino rats (200–300 g), fasted for 24 hr but with free access to water.

Isolation and culture conditions of rat hepatocytes. Hepatocytes were isolated as previously described [20]. Cell integrity was tested by Trypan blue exclusion and was between 82 and 92%. Hepatocytes were seeded at a density of 1.5×10^6 cells per 28 cm^2 petri dish in 4 mL medium. The latter consisted of 25% Medium 199 and 75% Minimum essential medium, supplemented with 1 mg/mL bovine serum albumin, 10 μg/mL bovine insulin, 10% fetal calf serum and antibiotics (penicillin 4.5 μg/mL, streptomycin sulphate 50 μg/m, and kanamycin monosulphate 50 μg/mL).

For cytochrome P450 measurements, 10^7 cells were plated per 175 cm^2 petri dish in 24 mL medium. The medium was renewed 4 hr after cell seeding and every day thereafter. Co-cultures were set up by adding after 4 hr, rat liver epithelial cells in order to get confluency [10]. These cells were obtained from 10-day-old Sprague–Dawley rat livers by trypsinization as described in detail by Williams *et al.* [21]. The epithelial cells were cultured in Williams' medium and were used before they underwent spontaneous transformation, i.e. between the 20th and the 30th passage. In fact, isolation and culture of the epithelial cells was carried out under identical conditions as described by Guguen-Guillouzo *et al.* [10] since the technique was learned in their laboratory. For every day renewal of the medium, hydrocortisone hemisuccinate was added in the same amounts as previously used by the Guillouzo team [10, 11]: 7×10^{-5} and 7×10^{-6} M to the media of conventional and co-cultures, respectively. For PB induction a final concentration of 2 mM, supplemented via the medium, was used.

It was added after cell attachment (4 hr) and every day thereafter.

Enzyme assays. ECOD and AE activities of freshly isolated hepatocytes were determined as described previously [22, 23]. For cultures and co-cultures, cell layers were washed with Krebs–Henseleit buffer and buffer supplemented with 10 mM glucose at 37° was added. The reactions were started by addition of 1 mL of a 7-ethoxycoumarin solution (0.19 mg/mL buffer) and 10 μL of an aldrin solution (10.95 mg/mL ethanol), respectively. The final concentrations of substrates were 500 and 120 μM, respectively. The reactions were either stopped immediately (blanks) or after 10 min (ECOD) and 40 min (AE) by rapidly scraping cells from the plastic and freezing them together with their supernatants in liquid nitrogen.

Thawed samples were further processed as described before [22, 23].

GST activity was determined spectrophotometrically with 1-chloro-2,4-dinitrobenzene (CDNB) as a general substrate [24].

The epithelial cells used in all the co-cultures have been examined for eventual enzymic activity and for their cytochrome P450 content. It appeared that under control conditions and after 14 days PB treatment (2 mM) no activity could be measured for ECOD and GST. For AE, trace activities were calculated from a very small peak, appearing with the same retention time as the dieldrin peak on the chromatograms. This was substracted from the activities measured for the co-culture samples. No cytochrome P450 could be detected in all the epithelial cell samples examined.

In order to be able to express the results obtained for co-cultures against hepatocyte proteins, the protein content of the hepatocytes and of the epithelial cells was determined as follows. After removing the medium, cells were washed with phosphate buffered saline (PBS) and total proteins were determined on three dishes. The same procedure was applied on another three dishes but, after washing, 4 mL of a 0.7 mg collagenase/mL PBS solution, warmed up at 37°, was added. Cultures were kept at 37° for about 30 min and from then on the detachment of the hepatocytes was followed under the microscope. The hepatocytes were removed, the remaining epithelials washed with PBS and their protein content was measured.

Cytochrome P450. After removing the medium, cells were washed, scraped off, homogenized and cytochrome P450 content was measured according to Omura and Sato [25] in 10,000 *g* supernatant using the conditions described by Paine *et al.* [26].

Protein assay. Proteins were measured using a "Bio-Rad protein assay kit" with bovine serum albumin as a standard.

Statistics. The results have been analysed using two-factor repeated measures analysis of variance (time, treatment) followed by paired *t*-tests, wherever suitable [27].

RESULTS

Cytochrome P450 content

In conventional cultures, with or without PB addition, the cytochrome P450 content decreases continuously as a function of time ($P < 0.001$) (Fig. 1a). After 1 week only 7% of the initial value is found back under control conditions. Daily supplementation of the medium with 2 mM PB tends to increase the cytochrome P450 content but both curves, shown in Fig. 1, are not significantly different (NS).

In co-cultures (Fig. 1b), the cytochrome P450 curves with and without PB treatment are significantly different ($P < 0.001$) and both show time dependency ($P < 0.001$). Under control conditions and after PB treatment, it is observed that, after an initial fall, the cytochrome P450 content is kept constant around 25 and 55% of the starting value, respectively, and this is true throughout the remaining culture period (paired *t*-tests between two time intervals not significant (NS) from 4 and 7 days on, respectively).

Enzymic activities

ECOD. The ECOD activity in conventional cultures is shown in Fig. 2a. It significantly decreases in function of culture time ($P < 0.001$). When PB is added, time dependency is not evident anymore as

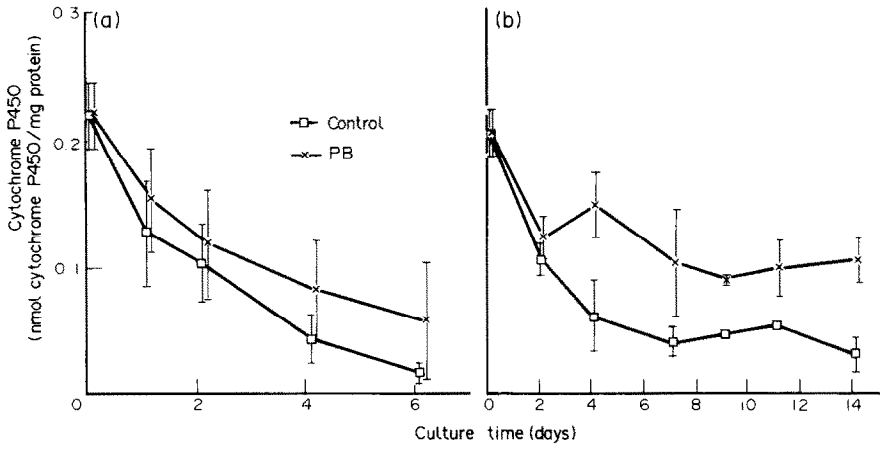

Fig. 1. Cytochrome P450 content of (a) conventionally and (b) co-cultured adult rat hepatocytes with and without PB (2 mM) treatment. The results are expressed as mean ± SD [(a) N = 5; (b) N = 7]. For co-cultures, the protein content of the epithelial cells is taken into account and the results are expressed against the hepatocyte proteins.

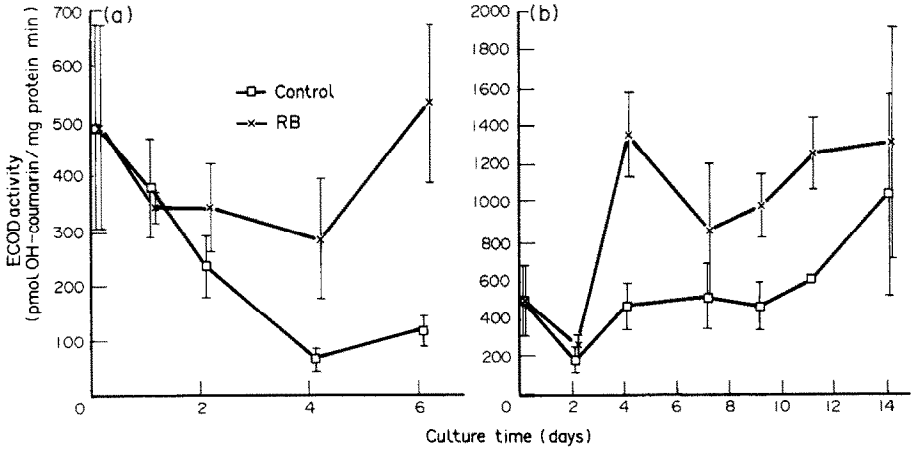

Fig. 2. ECOD activity of (a) conventionally and (b) co-cultured adult rat hepatocytes with and without PB (2 mM) treatment. The results are expressed as means ± SD [(a) N = 4; (b) N = 4]. For co-cultures, the proteins of the epithelial cells are taken into account and the results are expressed against the hepatocyte proteins.

shown by a two factor repeated measures analysis of variance with time and treatment as variables. A clear difference is observed between both curves ($P < 0.01$).

In co-cultures (Fig. 2b), time dependency is pronounced in PB treated cells ($P < 0.01$). There exists a significant difference between both curves ($P < 0.01$).

Under control conditions the ECOD activities remain close to that measured for freshly isolated hepatocytes (paired *t*-tests for the whole culture period are NS). After PB treatment, a significant increase (more than 100%) of the ECOD activity is observed after 4 days ($P < 0.05$) and this value is maintained throughout the culture period (paired *t*-tests between two time intervals are not significant from 4 days on).

AE. For conventional cultures (Fig. 3a), the AE activity decreases continuously in function of culture time ($P < 0.001$) and there exists no significant difference between the activity curves, obtained with and without PB treatment.

As far as the results for the co-cultures (Fig. 3b) are concerned, they differ completely from those obtained for the ECOD measurements (Fig. 2b).

It seems that the AE activity declines sharply within 2 days ($P < 0.001$) and no recovery is observed (all paired *t*-tests between two time intervals are NS from 2 days on). Eighty-five per cent of the initial activity is lost throughout the whole remaining culture period. Two mM PB has nearly no effect. Although one measures slightly higher AE activity values, both curves shown in Fig. 3 are not significantly different.

GST. In conventional culture (Fig. 4a), the GST activity curves, obtained under both conditions, are

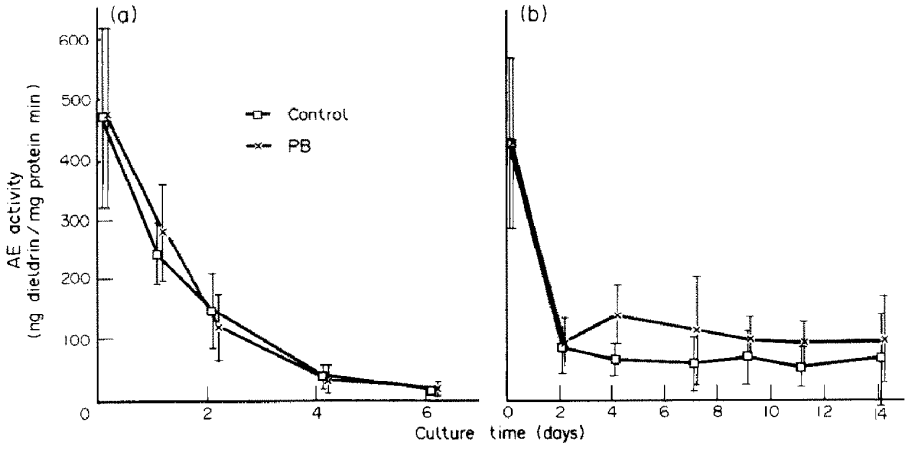

Fig. 3. AE activity of (a) conventionally and (b) co-cultured adult rat hepatocytes with and without PB (2 mM) treatment. The results are expressed as mean ± SD [(a) N = 5; (b) N = 7]. For co-cultures, the proteins of the epithelial cells are taken into account and the results are expressed against the hepatocyte proteins.

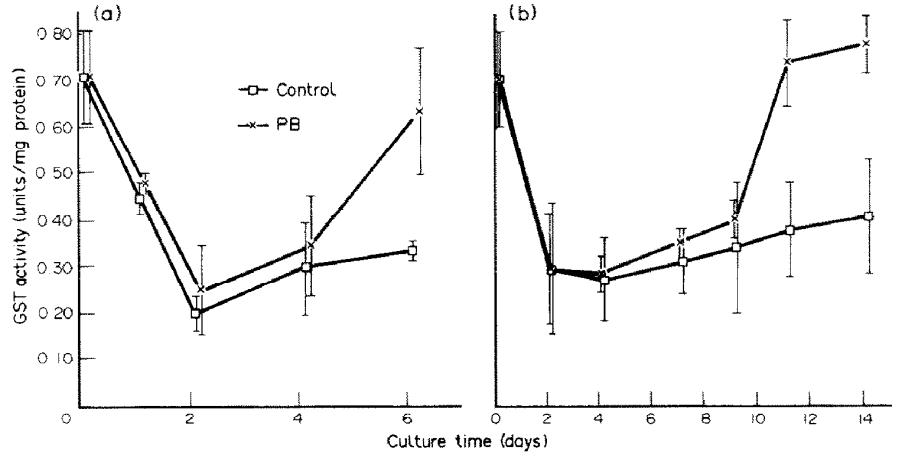

Fig. 4. GST activity of (a) conventionally and (b) co-cultured adult rat hepatocytes with and without PB (2 mM) treatment. The results are expressed as mean ± SD [(a) N = 3; (b) N = 3). For co-cultures, the proteins of the epithelial cells are taken into account and the results are expressed against the hepatocyte proteins.

generally spoken not significantly different and both show time dependency ($P < 0.01$). After an important decrease ($P < 0.01$), the GST activity remains constant at a level of 50% of the initial value for the rest of the culture period. When 2 mM PB is added only after 6 days a significant effect is measurable ($P < 0.05$).

As far as co-cultures (Fig. 4b) are concerned both GST activity curves differ significantly ($P < 0.05$) and both show time dependency ($P < 0.05$). After an initial decrease ($P < 0.01$), the GST activity remains constant under control conditions (all paired t-tests between two time intervals are NS from 2 days on) and increases significantly after PB treatment ($P < 0.05$ after 9 days).

DISCUSSION

From this study it was found that, in accordance with the literature, the total cytochrome P450 content [2, 3, 28] of conventionally cultured adult rat hepatocytes and the cytochrome P450 dependent enzymic activities, such as ECOD [29–31] and AE [32] decline continuously as a function of culture time. GST activity seems to be better maintained as has been reported earlier [33, 34].

As far as the effect of PB in conventional culture is concerned, it is known to have inducing effects on the cytochrome P450 content [35], the ECOD [29, 36, 37], the AE [8] and the GST [17] activities. In our conventional culture here, only the effect on ECOD could be shown statistically, although after PB treatment, the cytochrome P450 levels and the GST activities showed the expected tendency. It is important to note here that only 2 mM of PB was used whereas in the literature 3 mM or higher concentrations were often involved.

In co-cultures, it was found that, after an initial decrease for all parameters measured a steady state situation develops from 2 to 4 days on, this is after reaching complete confluency. The cytochrome P450 content, the ECOD, AE and GST activities are maintained from 3 to 4 days on at 25, 100, 15 and 50%, respectively, of their corresponding initial value, obtained for freshly isolated hepatocytes.

After PB treatment the parameters, mentioned above, were significantly increased with the exception of the AE activity, of which the inducibility seemed to be lost completely.

Comparison of these findings with results of the literature is rather difficult since only limited information is available concerning the biotransformation capacity of co-cultures of adult rat hepatocytes with epithelial cells. Bégué *et al.* [11] have stated that in co-cultures 100% of the cytochrome P450 content could be kept for at least 10 days together with the *N*-aminopyrine demethylation activity. Some other phase I and II enzymic activities have been reported to be maintained in co-culture too since drugs such as ketotifen [38], oxaminozoline and clonidine [39] were metabolized by various pathways. Maier [40] has reported that the AE activity in co-cultures of rat hepatocytes undergoes important decreases in function of culture time, especially when the results are compared with those of freshly isolated hepatocytes. He suggested that AE is a good parameter for dedifferentiation, more sensitive than other enzymic activities, which is confirmed by our findings. Data on GST activity in co-culture are only available from our group [17–19, 33, 41] and the effect of PB on phase I in this new culture system has not yet been documented until now.

From our results, it appears that co-cultures of rat hepatocytes maintain partially active cytochrome P450 for at least 10 days. Only a selective loss is observed. A constant situation is reached after complete confluency. In conventional culture no steady state occurs and all parameters measured, with the exception of GST, decrease continuously as a function of culture time.

The partial loss of the cytochrome P450 content in co-cultures is consistent with the observations made concerning the dramatical loss of AE expression and inducibility and the good maintenance of ECOD. Probably specific changes occur in the cytochrome P450 isoenzyme pattern during the first few days before complete confluency is reached. Indeed ECOD and AE are both mono-oxygenases, but dependent on different cytochrome P450 forms. ECOD depends especially on P450 IA_1 with P450 IIB_1, P450 IIA_1 and P450 IIB_2 giving much lower activities [29]. AE depends on P450, IIC_{11}, P450 IIC_6, P450 IID_1, P450 IIB_1, P450 $IIIA_2$, P450 IIA_1 and P450 IIB_2 with 450IIC_{11} and P450 IIC_6 giving the highest activity [12].

Clearly further study in this field is needed in order to find out which cytochrome P450 isoenzymes are involved in dedifferentiation and which factors could be of importance for their maintenance. Furthermore, a better knowledge of the cytochrome P450 isoenzyme pattern, especially in co-cultures, is a prerequisite for its use in biotransformation and toxicity studies of xenobiotics.

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