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CRUDE LIVER MEMBRANE FRACTIONS AS SUBSTRATE PRESERVE LIVER-SPECIFIC FUNCTIONS IN LONG-TERM, SERUM-FREE RAT HEPATOCYTE CULTURES

BASHAR SAAD, HANSPETER SCHAWALDER, AND PETER MAIER

Institute of Toxicology, Swiss Federal Institute of Technology and University of Zürich, CH-8603 Schwerzenbach, Switzerland

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Summary

Over time, rat hepatocytes cultured on collagen lose the capacity to express liver-specific functions. The influence on this degradation process of an alternative substratum—crude membrane fractions prepared from the liver of the same rat strain—was investigated. Freshly isolated rat hepatocytes were cultured in serum-free Williams E medium supplemented with aprotinin, selenium, dexamethasone, and insulin in flasks coated with a mixture of rat liver crude membrane fractions:collagen type I (100:1). The cells adhered firmly, exhibiting minimal spreading and remaining grouped in columns or in cell islands, and retained their liver-specific functions for more than 1 wk. Hepatocytes secreted substantially higher amounts of albumin than cells cultured on collagen-coated dishes, and on Days 1 and 9 in culture the total P-450 content was 72 and 40%, respectively, of that of freshly isolated cells. On Day 6, the 7-ethoxyresorufin-*O*-deethylase and the aldrin epoxidase activities were still more than 50% that of freshly isolated hepatocytes. Exposure to phenobarbital on Days 3 to 6 increased the total cytochrome P-450 content twofold; exposure to 3-methylcholanthrene increased the activity of the corresponding cytochrome P-450 isoforms to 20 times that observed in untreated cultures and 6 times that observed in freshly isolated cells. Thus, given the ease with which they are prepared, the use of crude membrane fractions combined with culture medium supplemented with aprotinin and selenium can facilitate the preparation of reproducible cultures suitable for long-term in vitro pharmacotoxicologic studies using rat hepatocytes.

Key words: adult rat hepatocytes; cytochrome P-450; albumin; EROD; aldrin epoxidase; crude membrane fractions; extracellular matrix; aprotinin.

Introduction

Rat hepatocyte cultures are an important tool for screening xenobiotics and elucidating the mechanisms involved in toxicity. Long-term cultures are often essential in the study of prolonged subtoxic exposures, adaptive responses, or the expression of toxic insults. However, under conventional culture conditions, adult rat hepatocytes lose most of their differentiated functions and xenobiotic metabolism within a few days. Therefore, a number of attempts have been made to increase the functional longevity of hepatocytes in primary culture. Three main approaches have been investigated: supplementing the culture medium with various components (Isom et al., 1985; Laishes and Williams, 1976a,b; Miyazaki et al., 1989; Mitaka et al., 1991; Williams et al., 1977); the use of more appropriate substrata (Michaelopoulos and Pitot, 1975; Michaelopoulos et al., 1979; Rojkind et al., 1980; Spray et al., 1987; Rubin et al., 1978; Schuetz et al., 1988; Shinji et al., 1988); and co-cultivation with selected liver epithelial cells which have already been cultured in vitro (Guillouzo et al., 1984; Guguen-Guillouzo et al., 1983).

Cell-to-cell and cell-to-substrate interactions, mediated by glycoproteins at the cell surface or in the extracellular matrix (for reviews, *see* Edelman et al., 1990), play an important role in the development, differentiation, and regeneration of multicellular organisms. The disruption of the liver tissue during the liver perfusion leads to a decline in the transcription of most liver-specific genes (Clayton et al., 1985). In vitro, cell-to-cell communication might be equally important for the maintenance of the functions of mature cells from adult tissues. At high cell densities, the reestablishment of cell-to-cell contacts between the isolated adult rat hepatocytes suppresses the growth observed at low cell densities and stimulates the expression of liver-specific differentiated functions, even in the presence of growth factors. This cell density-dependent reciprocal regulation between growth and function can be mimicked in low density cultures by the addition of cell membrane fractions containing a trypsin-sensitive factor (Nakamura et al., 1983). The establishment of heterotypic cell contacts between hepatocytes and nonparenchymal rat liver epithelial cells (RLEC) allows hepatocytes to regain their polarity and maintain their functional activities (Guguen-Guillouzo et al., 1983). Neither gap-junction communication nor soluble factors seemed to be involved (Mesnil et al., 1987). More recently it has been shown that plasma membrane proteins from these RLEC contribute to the cell contact-mediated regulation of liver-specific genes (Corlu et al., 1991).

The maintenance of an in vivo-like xenobiotic metabolism in hepatocytes is important for drug and toxicity testing in vitro. For such practical applications cultures must be easy to prepare, efficient, and reproducible. Therefore, in this study we explored the possibility that crude membrane fractions (CMF) from adult rat liver might mediate cell-to-cell interactions, allowing the preparation of cultures of adult hepatocytes that retain their functional xenobiotic metabolism over extended periods of time. We found that rat hepa-

tocytes cultured on CMF-coated flasks under serum-free conditions in a medium supplemented with the protease inhibitor aprotinin (Asami et al., 1984; Watanabe et al., 1987; Miyazaki et al., 1989) maintained their functional activities for more than 1 wk at levels between 40 and 80% of freshly isolated cells.

Materials and Methods

Preparation of Crude Membrane Fractions

Crude membrane fractions were prepared from male Sprague-Dawley rat liver (55 ± 5 days and 220 to 270 g body weight) by the following procedure: After perfusion with phosphate buffered saline (PBS) the liver was aseptically removed, quickly dissected, and homogenized on ice with 10 strokes of a sterile Dounce homogenizer in 50 ml hypotonic buffer (1 m*M* $NaHCO_3$, 1 m*M* $MgCl_2$, 0.2 m*M* $CaCl_2$, 0.2 m*M* spermidine, pH 7.9) containing protease inhibitors [5 μg/ml phenylmethylsulphonylfluoride, 1 mg/ml soybean trypsin inhibitor (Sigma, St. Louis, MO), 1 μg/ml turkey eggwhite trypsin inhibitor (Sigma), and 1 μg/ml iodoacetamide]. The homogenate was then kept on ice and stirred continuously for 1 h before centrifugation for 15 min at 2000 ×*g* and 4° C. The supernatant was collected and the residual pellet homogenized with a sterile Dounce homogenizer and centrifuged twice using the same procedure. The three batches of supernatant were mixed and further centrifuged for 15 min at 9000 ×*g* and 4° C. The resulting supernatant was centrifuged for 80 min at 30 000 ×*g* and 4° C. The pellet from this highspeed centrifugation contained the CMF. It was resuspended in 200 ml Ca^{2+}- and Mg^{2+}-free PBS (pH 7.4) by 10 strokes with a Dounce homogenizer in PBS buffer and stored at −80° C until use. The preparation was performed under a germ-free laminar air flow. For electron microscopy, pellets were fixed in 2.5% glutaraldehyde in 0.1 *M* Na-cacodylate at pH 7.4. The samples were postfixed in OsO_4 and embedded in epoxy resin. Sections were stained with uranyl acetate and lead citrate. Examinations were carried out with a JEOL 1200-EX electron microscope operated at 100 kV.

Coating of the Tissue Culture Flasks

Collagen type I coating. Twenty-four hours before the experiments, tissue culture flasks (NUNC) with an internal surface area of 80 cm^2 were coated with 4 ml of PBS solution containing 60 μg collagen type I(COL)/ml (Collagen type I porcine, Pentapharm, Basel, Switzerland) for 30 min at room temperature. After washing with PBS, the flasks were air-dried and stored at room temperature until use.

Crude membrane fractions/collagen coating. The crude membrane fractions (CMF) were thawed, homogenized by 10 strokes with a Dounce homogenizer, and centrifuged for 5 min at 4000 ×*g* and 4° C. The supernatants were diluted with PBS to final concentration of 60 μg protein/ml. COL was added to a concentration of 600 ng/ml. The tissue culture flasks were then coated with 4 ml of this solution overnight at 4° C. After being washed twice with PBS, the flasks were immediately used for hepatocyte cultures before the coating had dried.

Hepatocyte Cultures

Hepatocytes were obtained from male Sprague-Dawley rats (54 ± 2 days and 240 to 270 g body weight) by the modified version of the collagenase perfusion method described by Maier et al. (1991). Freshly isolated hepatocytes were enriched by three centrifugation steps (20 ×*g* at 4° C for 2 min). The first two centrifugation steps were carried out in wash buffer [Williams E medium supplemented with 10 m*M* HEPES, 2 m*M* L-glutamine, 100 n*M* insulin, and 2% fetal bovine serum (FBS)] and the third in wash buffer without serum. The viability of the cells, as determined by the trypan blue exclusion test, was more than 90%.

The enriched hepatocytes were suspended in a culture medium consisting of Williams E medium supplemented with 100 n*M* dexamethasone, 100 n*M* insulin, 30 n*M* selenium, 1 μg/ml aprotinin, and 2 m*M* L-glutamine and without phenol red (culture medium). The cells were plated onto tissue culture flasks (NUNC) coated with COL or CMF/COL at a density of 10^7 cells/80 cm^2 and maintained in 12 ml of culture medium at 37° C and 5% CO_2. For comparison, cells were also cultured under conventional conditions on COL-coated flasks in a culture medium without aprotinin and selenium and were kept for the first 4 h in culture medium supplemented with 2% FBS (COL/FBS). After 4 h and then every 24 h the medium was replaced with the same culture medium but without FBS.

Enzyme Induction by Phenobarbital and 3-Methylcholanthrene

After 3 days in culture, hepatocytes were maintained for another 3 days in the presence of 3 m*M* phenobarbital or 25 μ*M* 3-methylcholanthrene (MC) in dimethylsulfoxide (DMSO) (0.25% final concentration). The culture medium was renewed every 24 h. The effect of phenobarbital and 3-methylcholanthrene on enzyme activity was determined on Day 6.

Quantification of Albumin Secretion

The amount of albumin in the culture supernatant was measured using enzyme-linked immunosorbent assay (ELISA) as described by Saad et al. (1988). Briefly, the supernatant was diluted to 0.5% with PBS and incubated in 96-well microtiter plates (NUNC) for 1 h at 37° C, or overnight at 4° C. After washing in PBS, the nonspecific binding sites were blocked by incubation in PBS containing 0.5% bovine serum albumin (BSA) for 1 h at room temperature. After washing again with PBS, peroxidase-conjugated goat anti rat albumin antibody (Cappel, West Chester, PA) was added in PBS containing 1% BSA and the plates were incubated for 2 h at room temperature. The microtiter plates were then washed, the substrate (0.5 mg 2,2-azino-di-3 ethylbenzothiazoline-6-sulfonic acid per ml 100 m*M* Na-acetate, 50 m*M* Na-phosphate and 9×10^{-3}% H_2O_2) added and the absorption measured at 405 nm in an ELISA reader. All washing steps were carried out with PBS at room temperature. The amount of albumin was determined from a standard curve prepared by addition of a known quantity of rat albumin (Nordic-Immunology, Netherlands) to Williams E medium.

Background values were measured in the absence of culture supernatant and subtracted from the experimental values. All ELISA determinations were carried out in triplicate. For each data point (*see* Fig. 6), at least five independent experiments were carried out.

Quantification of Cytochrome P-450

The total cellular cytochrome P-450 content was measured as follows. Cell layers were washed with ice-cold PBS at pH 7.4, then scraped from the plate into a Dounce containing 2 ml PBS and homogenized on ice. The hemoprotein was then determined by difference spectrophotometry of the whole lysate as described by Bissell and Guzelian (1980).

Protein concentrations were determined according to Bradford (1976) using BSA as standard.

Lactate Dehydrogenase (LDH) Activity

The LDH activity in cell homogenates and in cell culture supernatants was determined at 24-h intervals spectrophotometrically (COBAS Fara) using a commercially available kit (Böhringer Mannheim), following the manufacturer's instructions.

7-Ethoxyresorufin De-Ethylase Activity (EROD)

The activity of EROD was determined in cell homogenates by fluorescence detection of 7-hydroxyresorufin as described by Burke et al. (1977).

Aldrin Epoxidase Activity

The aldrin expoxidase activity of freshly isolated and cultured hepatocytes was determined according to Lang et al. (1986). In brief, the culture medium was replaced and after equilibration for 30 min the cells were incubated with 50 μ*M* aldrin. After 30 min the cells were scraped from the

plates and sonicated. The dieldrin was extracted from the cell homogenates with hexane and analyzed by gas chromatography.

Results

CMF, Attachment, Protein, and LDH Release

Electron micrographs of CMF preparations showed a homogeneous plasma membrane fraction, with only occasional contaminations by lipid droplets or mitochondria. The morphologic appearance (Fig. 1) was comparable to rat hepatocyte plasma membranes as reported by Hubbard et al. (1983). The coating of substratum in the culture flasks influenced the attachment, morphology, and function of the hepatocytes. Hepatocytes cultured on COL with aprotinin/selenium in the culture medium (COL) or under conventional conditions (on COL and serum for the first 4 h; COL/FBS) attached and spread rapidly. Where dishes were coated with only crude membrane fraction and cultured in medium supplemented with aprotinin/selenium, hepatocytes attached to the substratum very slowly and loosely, leading to accidental loss of cells during daily medium exchange. However, the combination of CMF/COL in the ratio of 100:1 and aprotinin/selenium supplemented medium enhanced cell attachment considerably. Still hepatocytes attached more slowly to the substratum than with COL alone. They exhibited minimal spreading (Fig. 2 *B*), forming columns instead, in marked contrast to the flattened appearance of cells in COL cultures (Fig. 2 *A*). The morphologic differences persisted as the cultures aged. The hepatocytes cultured on CMF-COL remained grouped in columns or in cell islands.

The effect of the protease inhibitor aprotinin and selenium on viability and functions in COL and in CMF/COL cultures at Day 6 were investigated (Table 1). The presence of aprotinin and selenium in the culture medium in COL-coated flasks increased the total protein per flask and LDH activity by 4.5 and 7 times, respectively. The contribution of CMF without aprotinin/selenium increased the two parameters 2 and 3 times, respectively. The liver-specific functions involved in xenobiotic metabolism, however (cytochrome P-450), were equally well maintained in COL cultures with aprotinin/selenium as in CMF/COL cultures without aprotinin/selenium. The combination of the two (CMF and aprotinin/selenium) showed maximal improvements.

The protein content per dish was used as an indirect indicator of the number of cells available (a measure of attachment-detachment). Under conventional conditions (COL/FBS), the protein content decreased rapidly after 6 days (Fig. 3 *A*), whereas in CMF/COL or COL cultures, protein was about 8 to 10 mg per flask after 24 h, decreased to about 6 to 7 mg after 3 days, and remained at this level for up to 9 days.

The relationship between the protein content and the intracellular LDH activity served as a measure of the integrity of the hepatocyte plasma membrane. The LDH activity of freshly isolated hepatocytes was 2.1 ± 0.2 U/mg total protein. LDH activity in COL/FBS cultures decreased from Day 3 and reached levels of about 57 and 36% of the initial activity after 6 and 9 days, respectively (Fig. 3 *B*). In dishes coated with COL or with CMF/COL the LDH activity remained, respectively, at 70 to 90% and 90 to 100% of that of freshly isolated cells. At Day 6 the extracellular LDH activity as a percentage of the total activity was 9% in COL, 2.7% in CMF/COL, and 27% in COL/FBS cultures.

Cytochrome P-450 Content

The initial level of cytochrome P-450 in freshly isolated hepatocytes was 220 ± 10 pmol/mg of total protein. This value corresponds well with those reported by other investigators (e.g., Steward et al., 1985: 224 pmol/mg protein; Lindsay et al., 1991: 240 ± 30 pmol/mg). In rat hepatocytes cultured on CMF and maintained in culture medium (CMF/COL), the level of P-450 declined more slowly than in cells cultured under conventional conditions (COL/FBS) (Fig. 4). After 24 h the cytochrome P-450 content fell to 72% of the initial level and after 3 days to approximately 54%, reaching on Day 6 a level of approximately 40%, there remaining stable until the end of the experiments on Day 9 (Fig. 4). In hepatocytes cultured on collagen (COL), the reduction within the first 24 h was twice that observed in CMF/COL cultures. However, from Days 3 to 9 the evolution of the P-450 content paralleled, at 10% lower levels, that observed in CMF/COL cultures. In COL/FBS cultures the cytochrome P-450 content was relatively high over the first 24 h, but then fell rapidly, approaching zero at Day 9 (Fig. 4). No P-450 was detectable, even with immunologic methods in CMF/COL-coated flasks without cells (Saad et al., 1993).

Activity of Cytochrome P-450-Dependent Isoforms

7-Ethoxyresorufin O-de-ethylase activity. The initial activity of EROD (P450IA1/2) in freshly isolated hepatocytes was 4.8 ± 0.9 $nmol \cdot min^{-1} \cdot mg^{-1}$ total protein. In hepatocytes cultured on CMF/COL, EROD activity was more than 90% of initial activity for the first 3 days and then decreased to approximately 40 and 70% of the initial activity after 6 and 9 days, respectively. EROD activity in cells cultured on COL decreased more quickly than that in cells cultured on CMF/COL, stabilizing at 25% of the activity of freshly isolated cells from Day 6. In conventional cultures (COL/FBS) the activity rapidly decreased, falling to 30 and 11% of the initial value after 3 and 6 days, respectively, and was not measurable on Day 9 (Fig. 5 *A*).

Aldrin epoxidase activity. The effect of the substratum on the stabilization of aldrin epoxidase activity is shown in Table 2. The activity in freshly isolated cells was 660 ± 80 $pmol \cdot min^{-1} \cdot mg^{-1}$ total protein. After 24 h in culture, 16% of the activity was lost and no difference was found between hepatocytes cultured on COL and hepatocytes cultured on CMF/COL. However, after 6 days, at one third of the value of freshly isolated cells and less than half (46%) of the value after 24 h, the aldrin epoxidase activity of cells cultured on CMF/COL was twice as high as that of cells cultured on COL.

Albumin Secretion

The amount of albumin secreted into the culture medium is commonly used as a measure of the retention of original differentiated function by hepatocytes in culture. Albumin secretion by cells cultured on CMF/COL remained high over the whole 9-day period (Fig. 6). At Days 6 and 9, hepatocytes in CMF/COL cultures secreted 2 to 3 times more albumin than hepatocytes in COL/FBS cultures and 1.5 times more than hepatocytes in COL cultures.

Inducibility of Cytochrome P-450 and Isoform Activity

Cytochrome P450 content. The effects of phenobarbital on the induction of cytochrome P-450 are shown in Fig. 7. On Day 6, after

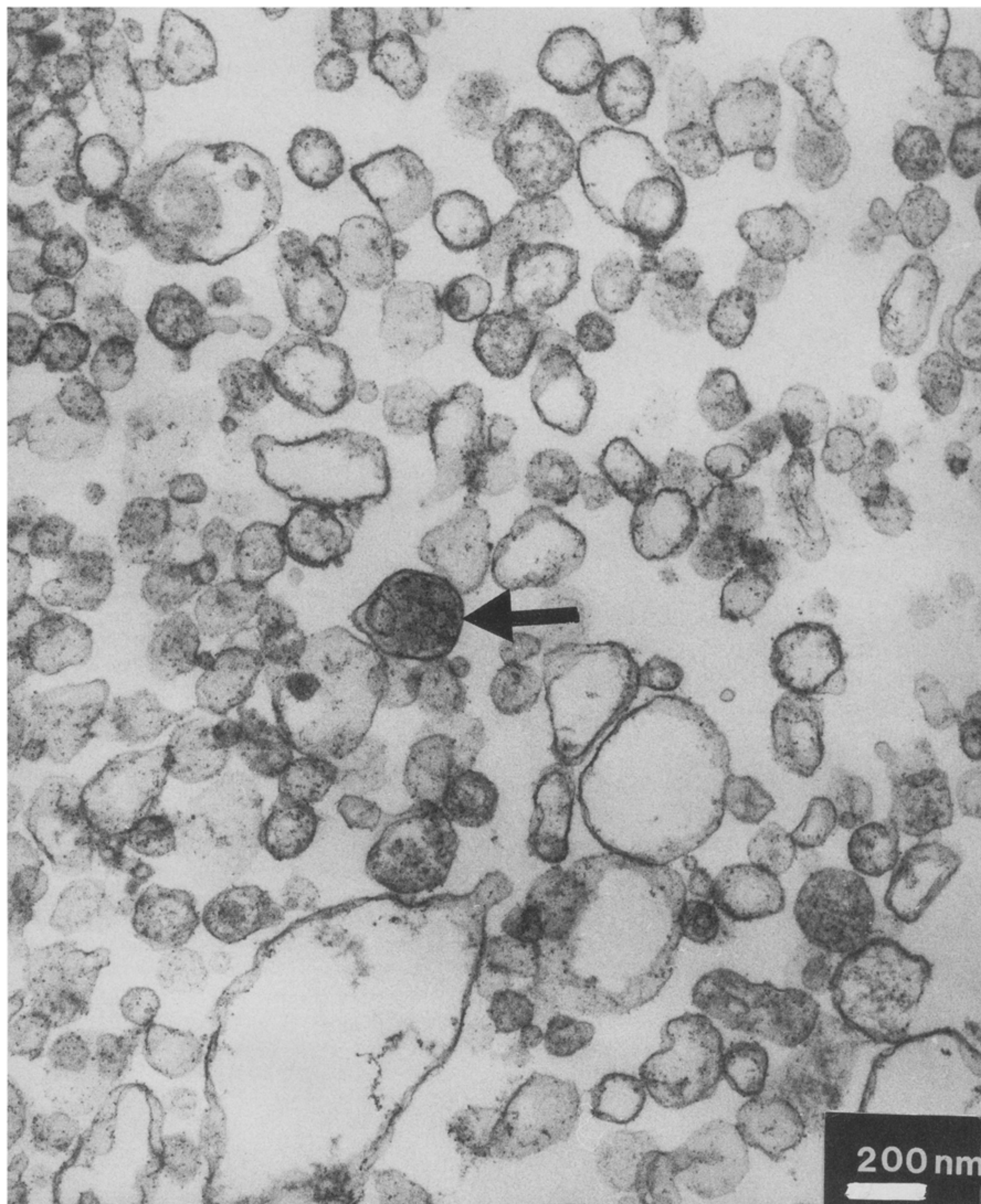


FIG. 1. Electron micrograph of crude membrane fractions. Vesicles of heterogeneous size are formed by bilayered membrane fragments. Contaminations by lysosomes, mitochondria, or lipid droplets were rarely seen. Some of the vesicles contain protein inclusions (*arrow*).

a 3-day exposure to 3 m*M* phenobarbital, the highest level of cytochrome P-450 content (1 nmol/flask) was measured in hepatocytes cultured on CMF/COL. The P-450 content was twice that observed in untreated hepatocytes. After induction, the total P-450 content in COL cultures was approximately 0.690 nmol and in COL/FBS cultures 0.540 nmol. The 3-day phenobarbital treatment increased the P-450 content in CMF/COL cultures to about 85%, in COL cultures to about 60%, and in COL/FBS cultures to about 30% of the P-450 content after 24 h.

7-Ethoxyresorufin-O-de-ethylase. The inducibility of 7-ethoxyresorufin O-de-ethylase activity by MC and phenobarbital is summarized in Fig. 5 *B*. As in the previous experiments, exposure lasted

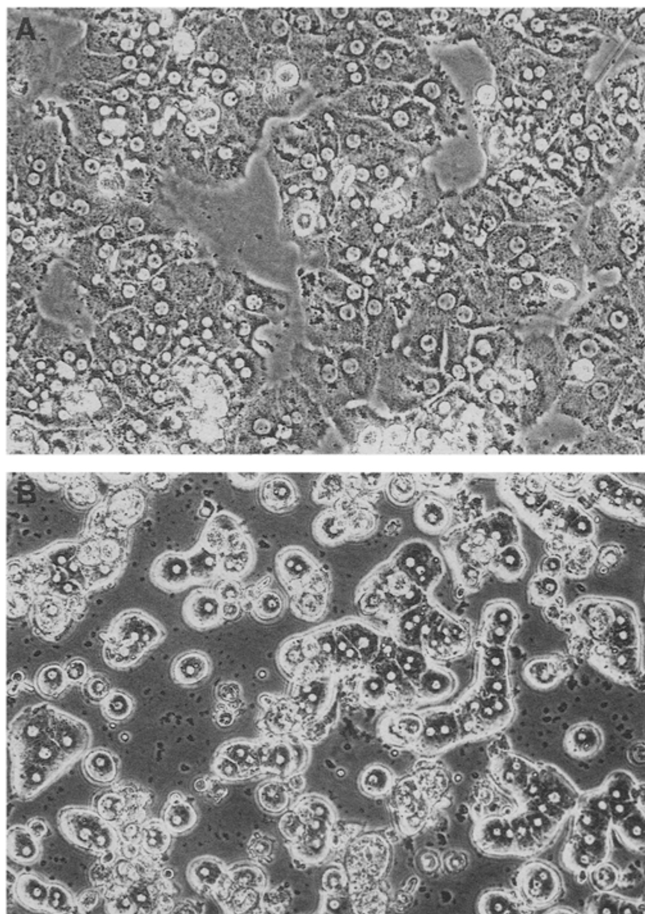


FIG. 2. Hepatocytes cultured for 24 h (*A*) on COL or (*B*) on CMF/COL (100:1)-coated culture flasks. In contrast to *A*, the hepatocytes in *B* are spherical, spread only minimally, and reaggregate to columns.

from Day 3 to Day 6 in culture. Analogous to the cytochrome P-450 content, the induction of enzyme activity by both MC and phenobarbital was again found to be highest in CMF/COL cultures. In CMF/COL cultures after 6 days, the EROD activity of the samples treated with MC was 20 times that observed in the untreated cultures and 6 times that observed in freshly isolated hepatocytes, clearly demonstrating the substrate specificity of the enzyme, because phenobarbital only doubled the EROD activity. DMSO caused no significant increase in activity. Hepatocytes cultured on COL in the absence of serum showed a very low response to all three substrates (Fig. 5 *B*).

DISCUSSION

The results from the present study indicate that the use of rat liver crude membrane fractions as a substratum in combination with a serum-free culture medium supplemented with aprotinin and selenium offers a significant improvement in the stabilization of xenobiotic metabolism in rat hepatocyte cultures over conventional culture conditions. The preparation of rat liver CMF is a relatively easy procedure that takes only a few hours. Hepatocytes cultured on a combined CMF/COL (100:1) substratum expressed substantially higher amounts of liver proteins, including cytochrome P-450 and secreted albumin, than hepatocytes cultured on a COL substratum. Aldrin epoxidase activity, for example (Table 2), a sensitive parameter for measuring dedifferentiation of cultured hepatocytes (Maier, 1988), was doubled at Day 6 compared to that observed in co-cultures with rat liver epithelial cells (Maier, 1988 and unpublished results; Rogiers et al., 1990). CMF maintained the adaptive response especially well, as shown after exposure of the cultured hepatocytes to 3-methylcholanthrene and phenobarbital from Days 3 to 6 (Figs. 5 *B* and 7).

Cell shape and the maintenance of liver-specific functions in long-term cultures are drastically affected by the substratum to which the cells are attached (Fig. 2). The morphology of hepatocytes cultured on CMF is very unusual. Rather than attaching individually and spreading rapidly, as on COL, the cells form irregular, three-dimensional structures, similar to those seen with other matrices, such as Matrigel (Schuetz et al., 1988), which enhance hepatocyte functions. By contrast, although hepatocytes attached efficiently to COL and remained viable, such cultures were unable to maintain the expression of many liver-specific functions over prolonged culture time, such as enzymes involved in xenobiotic metabolism. Similar problems have been encountered in cultures on components from extracellular matrix and membranes, including type IV collagen, laminin, and fibronectin (Bissel et al., 1987). The morphologic appearance of the cells plated on crude membrane fractions suggest that cell-to-cell interactions are enhanced under these conditions. Earlier studies with hepatocytes in culture provided evidence suggesting that aggregation per se preserves the expression

TABLE 1

EFFECT OF APROTININ AND SELENIUM ON THE VIABILITY AND FUNCTION OF HEPATOCYTES IN CULTURE

Culture medium	Total protein, mg/flask	Intracellular LDH		Albumin, µg/ml	P-450	
		U/mg protein	U/flask		pmol/mg protein	nmol/flask
COL						
Without aprotinin + selenium	1.47	1.03	1.52	24.0	0	0
With aprotinin + selenium	6.86	1.60	11.00	48.7	61	0.42
CMF/COL						
Without aprotinin + selenium	3.00	1.40	4.20	25.0	50	0.15
With aprotinin + selenium	6.27	2.00	12.50	62.2	83	0.52

[a] Cells were cultured at a density of 1.25×10^5 cells/cm^2 in tissue culture flasks coated with COL or CMF/COL and maintained in culture medium in the presence or absence of aprotinin/selenium for 6 days. Protein content, P-450 content, LDH activity, and albumin secretion per milliliter of culture medium were measured on Day 6.

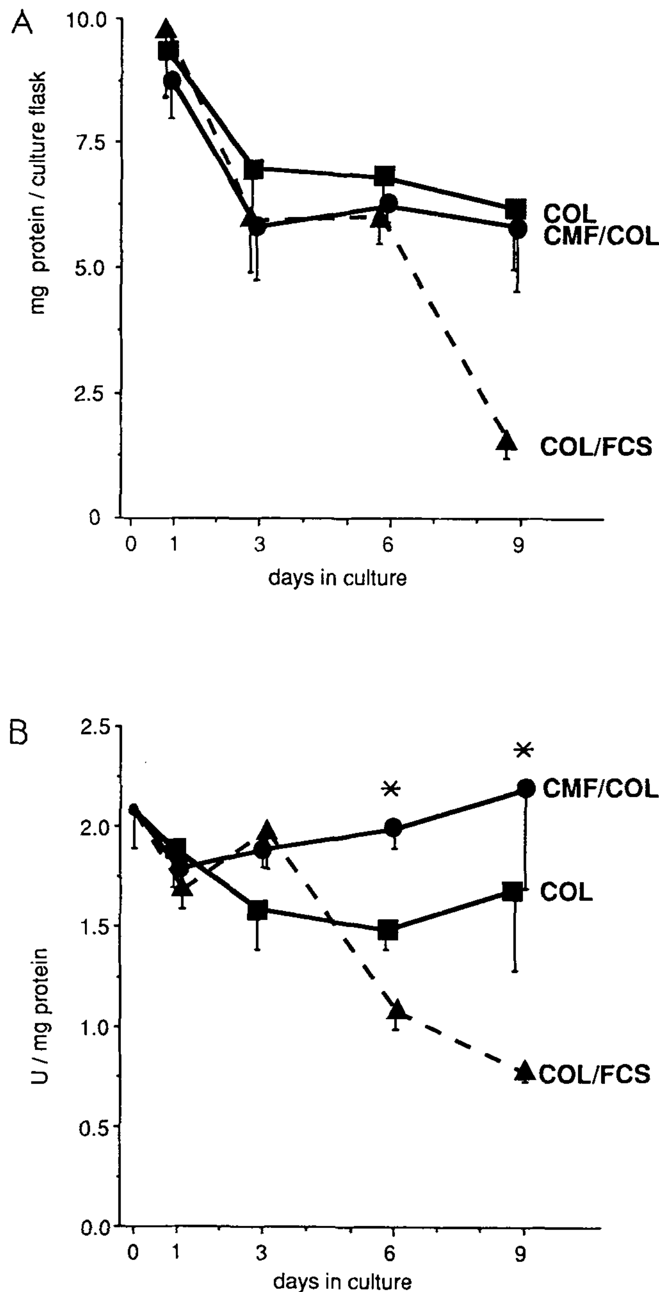


FIG. 3. *A*, influence of substratum and culture conditions on the total protein content for 80 cm^2 culture flasks and *B*, the intracellular LDH activity per mg protein; for hepatocytes (10^7 hepatocytes/flask) cultured on CMF/COL, on COL-coated culture flasks, and under conventional conditions (COL/FBS). LDH activities of freshly isolated hepatocytes (Day 0) were also determined. Values displayed represent the mean values ± SEM from five independent experiments. *Asterisk* significantly different from the corresponding values of COL-cultures at $P < 0.05$.

of differentiated functions (Koide et al., 1990). The data from the present study are not sufficient to determine whether the effects of crude membrane fractions are solely attributable to changes in cell shape, or whether the expression of some liver-specific functions is controlled by other mechanisms such as interactions with membrane proteins. The latter thesis is supported by the stabilization of xenobiotic metabolism observed in heterotypic co-cultures of rat epithelial-like cells and hepatocytes (Guillouzo et al., 1984). Cell-to-cell contact between hepatocytes and nonparenchymal rat liver cells, but not gap-junctions or soluble factors, has been shown to be essential for the expression of liver-specific functions in long-term cultures (Fraslin et al., 1985; Mesnil et al., 1987). Cell adhesion molecules may well be involved in this process because their influence in the peripheral nervous system on the activation of second messenger systems and on the cytoskeleton has been well documented (Schuch et al., 1989; Pollerberg et al., 1986). A recent report (Corlu et al., 1991) showed that a cell surface protein isolated from rat liver epithelial cells is involved in the maintenance of hepatocyte differentiation. Given that after collagenase perfusion only 30 to 50% of the cell surface molecules remain on the surface of the isolated cells (as shown for lectins by Weisz and Schnaar, 1991), hepatocytes depend on appropriate extracellular signals to stimulate hepatocyte-specific functions after perfusion. Probably, membrane proteins available in the crude membrane fraction are able to activate the intracellular pool of proteins involved in xenobiotic functions. It is possible that no single element of the extracellular matrix or membrane proteins is critical, but rather that various components must be present in appropriate in vivo-like ratios. CMF used in this study included membranes from all liver cells, from nonparenchymal and parenchymal cells. To maintain not only the composition but also the functions of the proteins, the membrane fraction was prepared without detergents and the coating on the culture flasks was not dried. Furthermore, possible trypsin-sensitive factors (Nakamura et al., 1983) were protected during preparation by the use of different trypsin inhibitors. All these measures will

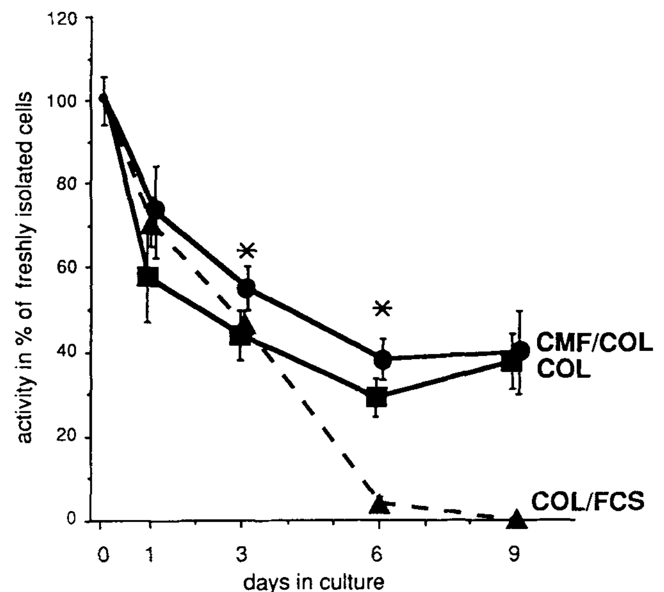
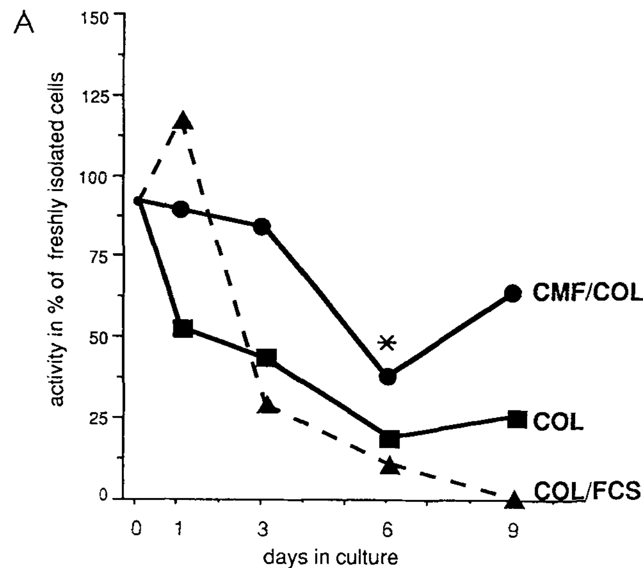


FIG. 4. Cytochrome P-450 content of cultured hepatocytes. Cells were cultured at a density of 1.25×10^5 cells/cm^2 in tissue culture flasks coated with CMF/COL, COL, or under conventional conditions (COL/FBS). Values displayed represent the cytochrome P-450 content as a percentage of the P-450 content of freshly isolated cells (0.22 nmol/mg of protein ± the SEM from at least five independent experiments carried out in duplicate. *Asterisk* significantly different from the corresponding values of COL-cultures at $P < 0.05$.



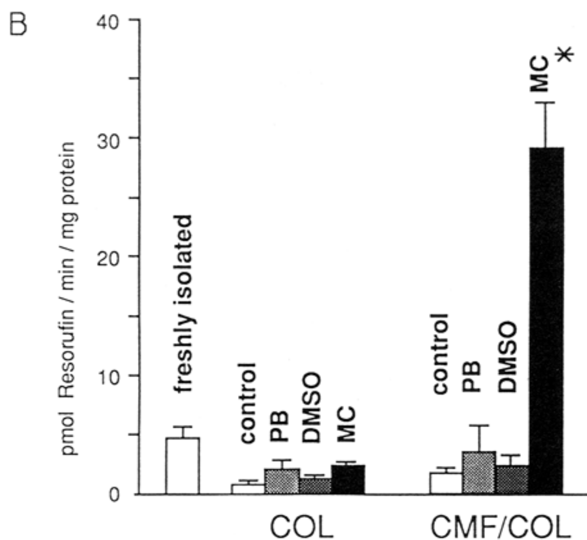


FIG. 5. *A*, activity and *B* induction of EROD in cultured hepatocytes. EROD activity was measured in cell homogenates from freshly isolated hepatocytes, from hepatocytes cultured under conventional conditions (COL/FBS), from hepatocytes cultured on COL-coated, and from hepatocytes cultured on CMF/COL-coated tissue culture flasks at densities of 1.25×10^5 cells/cm^2. Values displayed represent the mean values of the EROD activity from four independent experiments as the percentage of the activity in freshly isolated cells (*A*). Effect of phenobarbital (PB), MC, and DMSO on the activity of EROD was measured at Day 6 after daily treatment with PB, MC, or DMSO for 3 days. A comparison was made between the homogenates from cells cultured on COL and cells cultured on CMF/COL. Mean values (pmol/min/mg protein) ± SEM from four independent experiments are presented. *Asterisk* = significantly different from the corresponding values of COL-cultures at $P < 0.05$.

have helped to preserve the membrane components in their native state.

The results from this study support previous reports (Watanabe et al., 1987; Miyazaki et al., 1989) that aprotinin is essential if

TABLE 2

ALDRIN EPOXIDASE ACTIVITY OF HEPATOCYTES CULTURED ON CMF/COL AND ON COL WAS DETERMINED AFTER 24 H AND AFTER 6 DAYS IN CULTURE

	Aldrin-Epoxidase Activity pmol/min/mg protein	
	1 Day Culture	6 Day Culture
CMF/COL	422 ± 9	190 ± 30
COL	420 ± 20	100 ± 30

[a] Values given represent the values displayed in the graph (pmol/min and mg protein) ± SD from a total of four measurements from two independent experiments.

stable serum-free, long-term cultures are to be established. Serum itself contains cell attachment and spreading factors, including fibronectin and vitronectin (Hynes and Yamada, 1982; Hayman et al., 1985). Serum probably acts as a protease inhibitor but has also been found to inhibit the expression of liver-specific functions in cultured hepatocytes (Enat et al., 1984). In our approach, the protective effect of serum was introduced during the hepatocyte enrichment procedure. It was restricted to an interval of not more than 10 min at 4° C, at a stage where the metabolic activity of the hepatocytes had ceased.

In conclusion, serum-free cultures of hepatocytes on flasks coated with CMF combined with culture medium supplemented with aprotinin and selenium provide a simple and easily reproducible culture system to support the expression and inducibility of hepatic

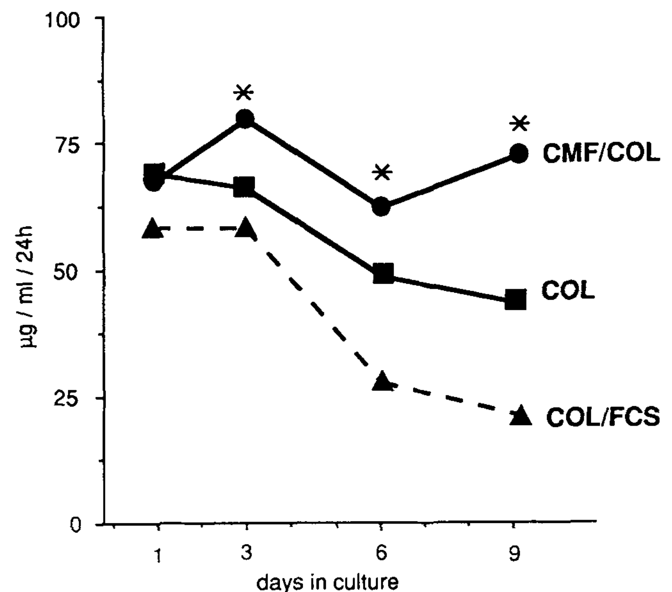


FIG. 6. The secretion of albumin by cultured hepatocytes. Cells were cultured on CMF/COL or on substratum COL. Comparison was made with hepatocytes cultured under conventional conditions (COL/FBS). At the indicated time, the amount of albumin in the culture medium was determined in ELISA as described in Materials and Methods. Values displayed represent the amount of albumin (µg/ml, 24 h) from five independent experiments carried out in duplicate. *Asterisk* = significantly different from the corresponding values of COL-cultures at $P < 0.05$.

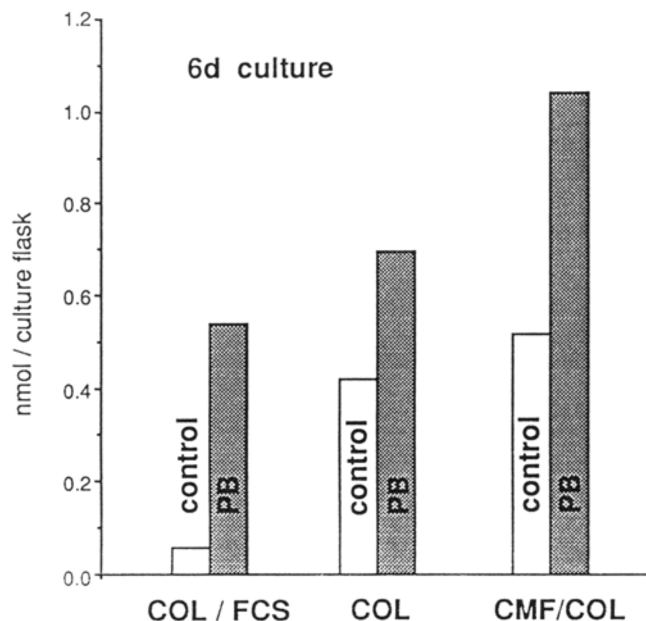


FIG. 7. The inducibility of cytochrome p-450 by pb. effect of pb on the expression of p-450 was measured on day 6 after treatment with 3 mm PB for 3 days. Cells were cultured at a density of 1.25×10^5 cells/cm^2 in tissue culture flasks on COL, on CMF/COL, and under conventional conditions (COL/FBS). Values given represent the mean from one representative experiment out of two independent experiments carried out in triplicate.

functions in rat hepatocytes. This system is particularly useful for routine testing of the adverse effects of drugs, chemicals, and carcinogenic agents.

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