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Maintenance of differentiated rat hepatocytes in primary culture

(chemically defined medium/dimethyl sulfoxide/albumin/plasma proteins)

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ABSTRACT **Normal adult rat hepatocytes remained viable and functional for at least 43 days when plated on collagen-coated dishes and fed chemically defined medium supplemented with dimethyl sulfoxide (Me_2SO). Hepatocytes isolated by collagenase perfusion and cultured in the presence or absence of Me_2SO were (*i*) examined by light and electron microscopy for morphological changes; (*ii*) analyzed for the production of albumin and other plasma proteins; and (*iii*) tested by autoradiography for DNA synthesis. Me_2SO-treated cells continued to produce specific plasma proteins during the entire culture period; albumin production was consistently high (11–19 μg/ml of culture medium per 24 hr) from day 2 to at least day 43 after plating. Ultrastructural analyses demonstrated that Me_2SO-treated hepatocytes resembled those from intact liver in organization of cytoplasmic organelles and cellular junctions. The optimal concentration for observing the morphological and biochemical effects of Me_2SO was 2% (vol/vol). We conclude that supplementation of chemically defined medium with Me_2SO enables maintenance of differentiated hepatocytes in culture for extended periods of time.**

Dimethyl sulfoxide (Me_2SO) is a dipolar aprotic solvent that is active in biological systems (1). Addition of 1–2% (vol/vol) Me_2SO to the culture medium of Friend virus-induced murine erythroleukemia (MEL) cells for 4–5 days causes 90% of the cells to express characteristics associated with normal erythroid differentiation, including alterations in morphology (2), induction of α- and β-globin synthesis (3, 4), and loss of the capacity for cell division (5). Me_2SO-induced differentiation has also been observed in a human leukemia cell line (6) and in cultured fibrosarcoma (7), neuroblastoma (8), human colon carcinoma (9), human lung cancer (10), and murine embryonal carcinoma (11, 12) cell lines.

Past efforts to achieve long-term culture of differentiated normal adult hepatocytes have not been successful. Limited proliferation and maintenance of adult hepatocytes can be achieved by supplementing culture medium with serum from partially hepatectomized animals (13) or by plating hepatocytes on liver extracellular matrix and maintaining them in serum-free hormonally defined medium (14). Proliferation also can be achieved by culturing hepatocytes at low cell density in the presence of insulin and epidermal growth factor (EGF), but maintenance of hepatocyte-specific characteristics requires high density or supplementation with hepatic plasma membrane material (15, 16).

In the present study, we employed a collagen-coated surface and supplemented the culture medium with Me_2SO in an attempt to extend the time *in vitro* that hepatocytes remain biochemically and morphologically differentiated. The addition of Me_2SO had a dramatic effect; hepatocytes retaining morphological and biochemical characteristics of normal liver could be maintained in culture for as long as 43 days. Note that Me_2SO, used previously to induce differentiation in tumor cells (2, 6–12, 17–20), is used here to maintain differentiation of a normal cell.

MATERIALS AND METHODS

Preparation of Hepatocyte Cultures. Hepatocytes were isolated by *in situ* collagenase perfusion of male Fischer F344 rats (180–200 g; Charles River Breeding Laboratories) as previously described (21) and modified (22, 23). Cells were used only when the viability was 90% or greater. Hepatocytes were washed in L-15 medium supplemented with 5% fetal calf serum (23) and plated at a density of 10^6 cells per culture on 60-mm plastic cell culture dishes coated with rat tail collagen (24). Plating efficiency was 90–98%. At 6 hr after plating, hepatocyte monolayers were fed a chemically defined medium termed HCD, a serum-free medium supplemented with bovine serum albumin, insulin, dexamethasone, glucagon, and transferrin as previously described (25). At 20–24 hr after plating, cultures were fed unsupplemented HCD medium or HCD medium supplemented with Me_2SO or phorbol 12-myristate 13-acetate (PMA) and fed every 24 or 48 hr for the remainder of the experiment. For measuring rat albumin concentration, medium samples were always taken 24 hr after feeding. Parenchymal and nonparenchymal cells were identified by phase-contrast light microscopy.

Radiolabeling of Secreted and Intracellular Proteins. Proteins secreted by hepatocyte monolayers were radioactively labeled as previously described (26, 27) except that the cells were radiolabeled for 6 hr at 37°C with 2 ml of serum-free medium containing [^{35}S]methionine (Amersham) at 50 μCi/ml (1 Ci = 37 GBq) as the only methionine in the medium. Intracellular proteins from hepatocytes were radioactively labeled and incorporation into trichloroacetic acid-precipitable material was measured (26).

Immunoprecipitation of Albumin, α-Fetoprotein (αFP), and Plasma Proteins. Antibody to α-FP purified from amniotic fluid was prepared as previously described (26). Antibodies to purified rat plasma proteins and rat immunoglobulin were purchased (Cappel Laboratories, Westchester, PA). Immunoprecipitation was performed by allowing radioactively labeled medium to react with specific antibodies followed by adsorption to staphylococcal protein A (IgGsorb, The Enzyme Center, Boston, MA) as described (26, 27). Immunoprecipitates were analyzed on 10–18% polyacrylamide gradient gels.

Albumin Quantitation. The amount of rat albumin secreted into the medium was measured by rocket immunoelectrophoresis as described (28). Rat serum albumin (fraction V; Sigma) was diluted in culture medium (with or without Me_2SO and with or without PMA) and used as a standard. Goat antibody to rat albumin was purchased from Cappel

Abbreviations: αFP, α-fetoprotein; Me_2SO, dimethyl sulfoxide; EGF, epidermal growth factor; HCD, chemically defined medium; MEL cells, Friend virus-induced murine erythroleukemia cells; PMA, phorbol 12-myristate 13-acetate.

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Laboratories. Medium from freshly isolated hepatocytes was included in each gel to serve as an additional positive control.

Autoradiography to Detect Percentage of Cells Synthesizing DNA. Hepatocytes on collagen-coated dishes were labeled with 5 μCi of [^{3}H]thymidine (42 Ci/mmol, Amersham) per ml for 5 hr, rinsed three times in phosphate-buffered saline, and treated with neutral buffered 10% Formalin for 1 hr. The dishes were coated with 50% (vol/vol) NTB-2 nuclear track emulsion (Eastman Kodak) and exposed at 4°C for 1 week. Autoradiograms were treated with D-19 developer followed by Kodak fixer for 5 min each. The hepatocytes were lightly stained with Harris hematoxylin to identify intact cells and to visualize nuclei. A minimum of 1000 cells was counted to determine percent of labeled cells.

Electron Microscopy. Hepatocyte cultures were fixed for electron microscopy *in situ* with 2.5% (vol/vol) glutaraldehyde in 110 mM 1,4-piperazinediethanesulfonic acid (Pipes) buffer (29), pH 7.2, with 1.3 mM $CaCl_2$ and 5 mM $MgCl_2$, postfixed after a buffer wash with 1% osmium tetroxide in the same buffer, dehydrated through a graded series of ethanol solutions, and embedded in Epon 812.

RESULTS

Effect of Me_2SO on Culture of Hepatocytes *in Vitro*. When hepatocytes were placed in monolayer and maintained *in vitro* in HCD medium, parenchymal cells were identified for several days, but by 10–14 days in culture the cells deteriorated. When hepatocytes were maintained in HCD medium supplemented with 2% (vol/vol) Me_2SO, parenchymal cells began to orient in cords by 10–14 days. We previously showed that HCD medium reduced but did not eliminate the outgrowth of nonparenchymal cells on collagen-coated surfaces (25). It was apparent by 14 days after plating that the addition of Me_2SO eliminated more than 95% of the nonparenchymal cells. By 23 days in culture, the effect of Me_2SO-supplemented medium on maintenance of parenchymal cells was readily observable (Fig. 1*A*); approximately 50% of the plating surface contained islands of cells that had the same size and shape as hepatocytes in monolayer for only 2 days. In addition, each island contained one or more areas of apparently degenerated cells. In untreated cultures, occasionally, an area of three or four healthy parenchymal cells was observed. By 40 days after plating, cells occupied 70–80% of the collagen-coated surface (Fig. 1*B*) and the cell groupings contained one to three rows of elongated cells at the border (Fig. 1*C*). In contrast, no viable parenchymal cells remained in untreated cultures.

At 23 days after plating, hepatocytes fed medium containing 1% or 0.5% Me_2SO yielded parenchymal cells with morphology improved in comparison with those with no Me_2SO supplementation, but the cells appeared smaller than 2% Me_2SO-treated cells and were arranged in islands that in general lacked the well-defined borders observed for 2% Me_2SO-treated cells (data not shown). In addition, as the Me_2SO concentration was reduced, the number of nonparenchymal cells increased. The morphological effects of 2% Me_2SO supplementation were equally apparent whether cells were plated at 5×10^5 or 1×10^6 cells per 60-mm dish (data not shown).

DNA Synthesis in Me_2SO-Treated Hepatocytes. When Me_2SO-treated hepatocytes were subjected to autoradiography 43 days after plating, nuclei from 2.2% of the cells were labeled (Fig. 1*D*). Cells with labeled nuclei were equally prevalent at the periphery and in the central portion of the cell islands, indicating that DNA synthetic capacity was independent of cell location.

Proteins Secreted by Hepatocytes Cultured in Me_2SO-Containing Medium. A comparison of the profile of proteins secreted by Me_2SO-treated hepatocytes in culture for 28 days with that of untreated freshly isolated hepatocytes led to the following observations (Fig. 2*A*). (*i*) It was possible to identify 17 distinct bands of proteins with molecular masses of 9.5 to approximately 176 kDa in medium from either culture, with a prominent 69-kDa albumin band. (*ii*) The band migrating at approximately 17 kDa was absent from Me_2SO-treated cultures. This band, present only in medium from hepatocytes isolated from male rats, is most likely α_{2u}-globulin and is not expressed because of the absence of testosterone and other hormones in HCD medium. The profile of secreted proteins after 43 days was comparable to that seen after 28 days of Me_2SO treatment (Fig. 2*A*).

Radioactively labeled medium from 28-day (Fig. 2*B*) and 43-day (Fig. 2*C*) cultures was immunoprecipitated with specific antibodies to determine which plasma proteins were produced. Hepatocytes in Me_2SO-supplemented medium continued to produce plasma proteins secreted by differentiated adult hepatocytes (albumin, hemopexin, transferrin, etc.). We did not detect albumin, hemopexin, transferrin, or other plasma proteins in medium from hepatocytes in non-Me_2SO-supplemented cultures. αFP, an oncodevelopmental liver protein found normally in the yolk sac and fetal plasma, and which can reappear in the plasma of animals bearing hepatomas, was not detected in the medium of Me_2SO-treated hepatocytes in culture for 28 or 43 days (Fig. 2 *B* and *C*).

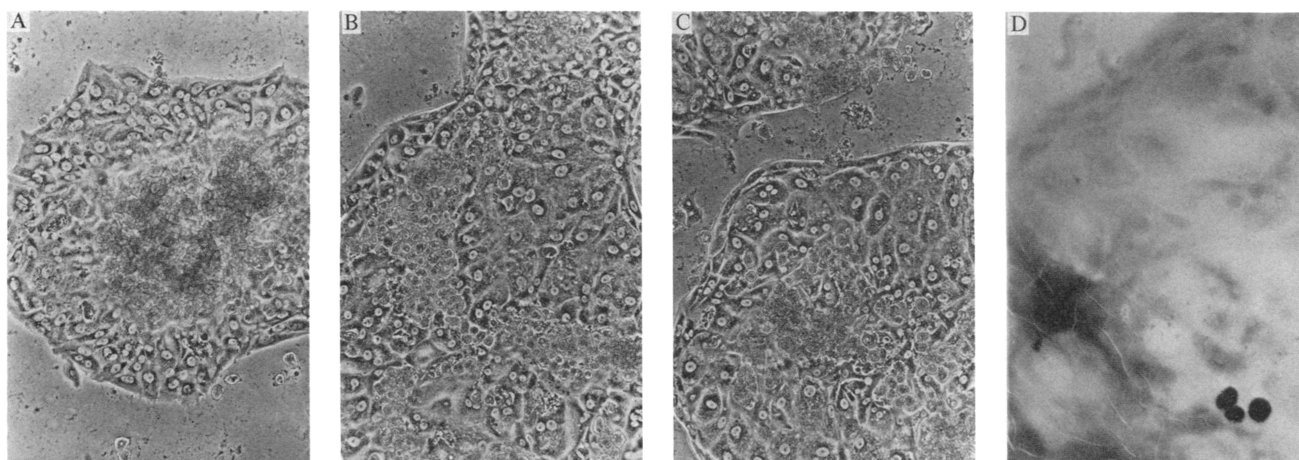

FIG. 1. Effect of Me_2SO on morphology of hepatocytes. (*A–C*) Photomicrographs of hepatocytes fed HCD medium supplemented with 2% Me_2SO and photographed 23 (*A*) or 40 (*B* and *C*) days after plating. In *C*, note the rows of elongated cells at the border of the cell island. (*D*) Autoradiogram showing labeled nuclei from hepatocytes fed HCD medium supplemented with 2% Me_2SO for 43 days. (*A–C*, ×88; *D*, ×145.)

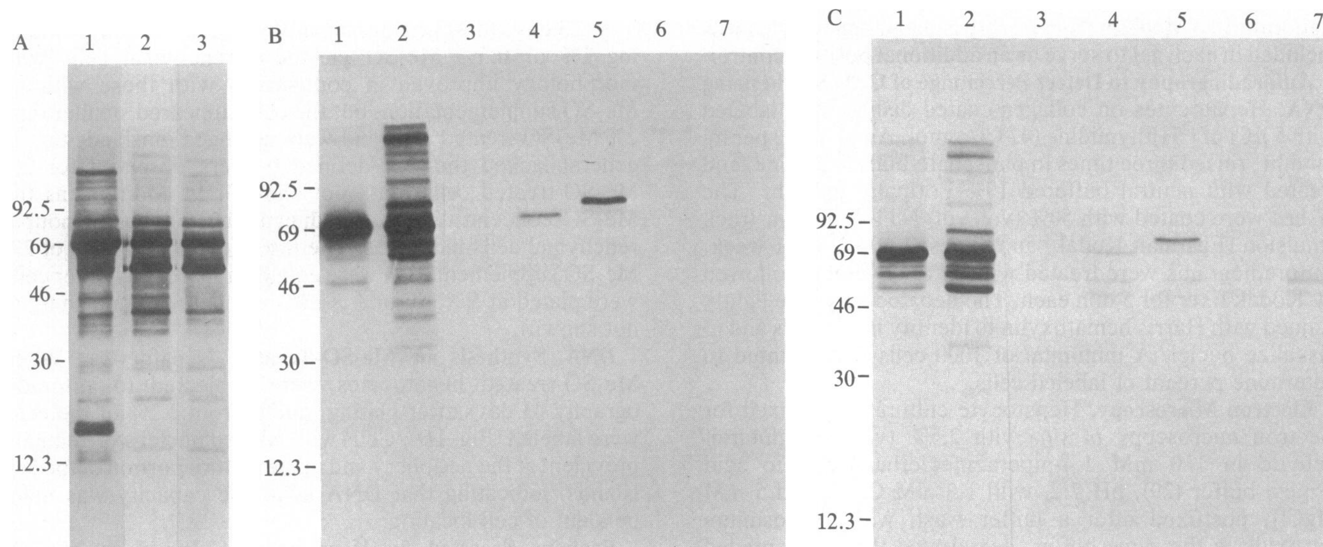

FIG. 2. Effect of Me_2SO treatment on protein secretion by hepatocytes. (*A*) Freshly isolated hepatocytes (lane 1) and Me_2SO-treated hepatocytes in culture for 28 days (lane 2) or 43 days (lane 3) were labeled with [^{35}S]methionine for 6-hr intervals and culture medium was collected. Equal volumes of radioactively labeled medium (30 μl) were loaded on each gel lane and subjected to electrophoresis in 10–18% polyacrylamide gradient gels. Positions of marker proteins of known molecular mass (kDa) are indicated on the left. (*B*) Radioactively labeled medium from hepatocytes in culture for 28 days and fed Me_2SO-supplemented HCD medium was immunoprecipitated with antibody to rat albumin (lane 1), plasma proteins (lane 2), plasminogen (lane 3), hemopexin (lane 4), transferrin (lane 5), αFP (lane 6), or immunoglobulin (lane 7). Overexposure indicates a faint plasminogen band in lane 3. (*C*) Radioactively labeled medium from hepatocytes in culture for 43 days and fed Me_2SO-supplemented HCD medium was immunoprecipitated with antibody to rat albumin (lane 1), plasma proteins (lane 2), plasminogen (lane 3), hemopexin (lane 4), transferrin (lane 5), αFP (lane 6), or immunoglobulin (lane 7).

Effect of Me_2SO on Albumin Production by Hepatocytes. In cultures fed HCD medium without Me_2SO, maximum albumin production (12–15 μg/ml of culture medium per 24 hr) was observed 4–6 days after plating (Fig. 3). By 10 days, a marked decline in albumin production was seen, and by 12–15 days albumin levels reached 2–3 μg/ml of culture medium per 24 hr. In most cultures, no albumin was detectable by 25 days after plating. In Me_2SO-treated cultures, albumin production persisted at high levels (11–19 μg/ml of culture medium per 24 hr); although medium collection experiments were terminated after 43 days, there was no indication that albumin production was declining.

Albumin level has been reported in quantities of μg/ml of culture medium per 24 hr. It was important to determine the amount of albumin per cell. Although accurate cell counts could not be obtained for non-Me_2SO-treated cultures because of the number of nonparenchymal and degenerated parenchymal cells, accurate counts could be obtained for

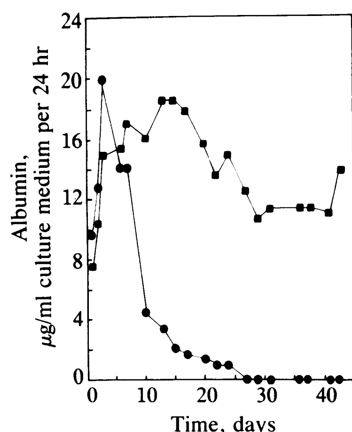

FIG. 3. Effect on Me_2SO on albumin production by hepatocytes. Hepatocytes were fed HCD medium (●) or HCD medium supplemented with 2% Me_2SO (■). Culture medium was collected 24 hr after cell feeding and analyzed for albumin content.

Me_2SO-treated cultures. At 43 days after plating, Me_2SO-treated cultures contained 6–7 $\times$ 10^5 cells per 60-mm plate when the initial plating density was 10^6 cells, indicating that at most 40% of the cells were lost or that greater than 40% of the cells were lost but new cells appeared. At 43 days after plating, Me_2SO-treated cells secreted 81.2 μg of albumin per 10^6 cells per 24 hr.

Ultrastructure of Me_2SO-Treated Hepatocytes. When thin sections of hepatocyte monolayers maintained in 2% Me_2SO-supplemented HCD medium for 37 days were examined by electron microscopy, the orientation of cells with regard to each other and overall cell shape seen at the light microscope level were verified. Examination at higher magnification showed that the cells contained large numbers of mitochondria and extensive rough and smooth endoplasmic reticulum, indicating that the cells were intact and metabolically active (Fig. 4*A*). The organelles were well organized within the cytoplasm, and desmosomes, characteristically found between hepatocytes in liver, were also seen (Fig. 4*B*). The cells also contained regularly shaped nuclei with prominent nucleoli and had a low nucleus-to-cytoplasm ratio, in contrast to the irregularly shaped nuclei and increased nucleus-to-cytoplasm ratio observed in transformed liver cells and hepatoma cells (30). Abnormalities, such as bundles of filaments in the cytoplasm and large, irregularly shaped mitochondria, which have not been observed previously by ultrastructural analyses of hepatocytes, were seen in some Me_2SO-treated hepatocytes; abnormal mitochondria have also been observed in Me_2SO-treated rat mammary glands (31). A limited number of cells contained a few autophagic vacuoles, indicating some degree of cell senescence.

Effect of PMA on *in Vitro* Culture of Hepatocytes. We also tested the ability of the tumor promoter PMA to facilitate maintenance of differentiation because PMA induces differentiation in other systems (32, 33). PMA-treated hepatocytes showed no differences by light microscopic observation from cells fed HCD medium without drug, and albumin production by PMA-treated cultures paralleled that of the untreated controls (data not shown). At 28 days after plating, cultures were radioactively labeled and incorporation into

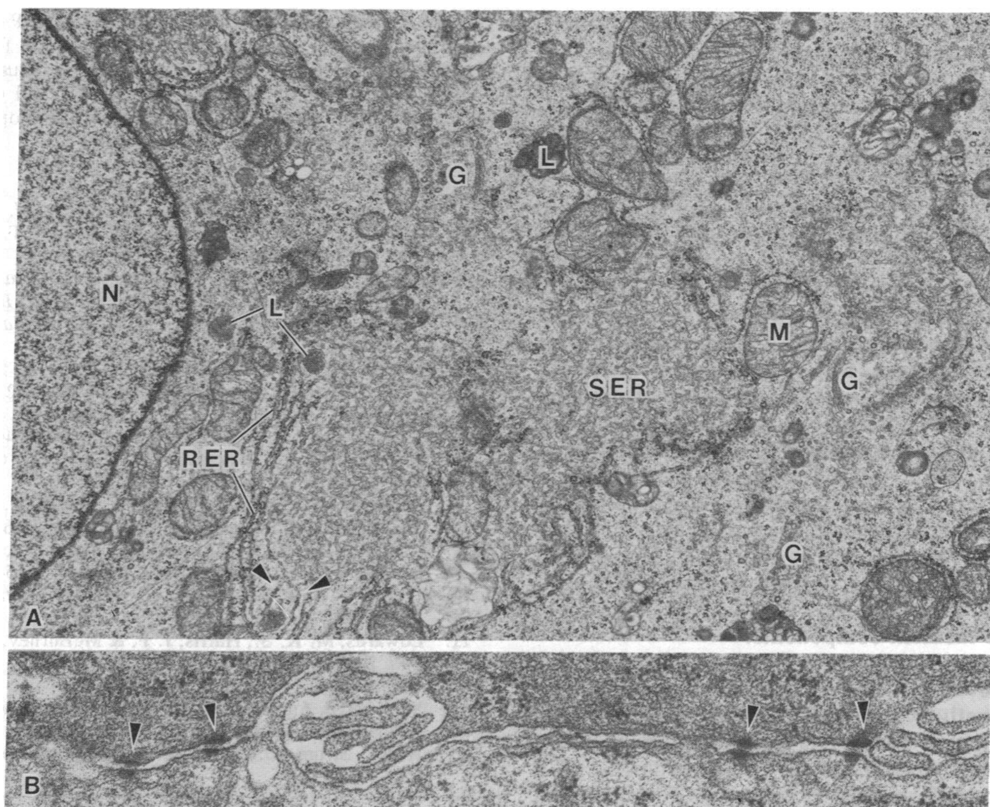

FIG. 4. Electron micrographs of Me_2SO-treated rat hepatocytes 37 days after plating. Cells were sectioned parallel to the surface of the dish. (*A*) Micrograph of a single cell, demonstrating the normal appearance and relationship of organelles and inclusions. N, nucleus; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum; M, mitochondrion; G, Golgi complexes; L, lysosomes. Continuity between SER and RER is shown (arrowheads). (×12,400.) (*B*) Desmosomes (arrowheads) located between the plasma membranes of opposing cells. (×34,800.)

secreted and intracellular protein was measured (Table 1). No significant differences were observed in PMA-treated compared with untreated cultures, but total incorporation of radioactivity into intracellular and secreted protein increased in Me_2SO-treated cells compared with control or PMA-treated cultures.

DISCUSSION

Liver is a system in which normal differentiation and malignant growth have been characterized. Recent investigations have shown that Me_2SO treatment of mouse and rat hepatoma cells results in loss of the ability to clone in soft agar medium (17), increased albumin production with a corresponding decline in γ-glutamyl transpeptidase expression (18), increased transferrin production (19), and a decline in the percentage of cells in the diploid G_1 population with a corresponding increase in the proportion of cells in either a G_2 or tetraploid G_1 population (20). To our knowledge, the use of Me_2SO to maintain differentiation in normal hepatocytes has not been reported prior to this study.

The effects of Me_2SO on long-term culture of hepatocytes are dramatic. Light microscopic observation of Me_2SO-treated cells after 40 days in culture indicated that the cells remained viable and were differentiated; this was substantiated by ultrastructural and biochemical analyses. Culture of morphologically intact albumin-producing rat (34) or human (35) hepatocytes for this length of time has been achieved previously, but only when hepatocytes were cocultured with

Table 1. Effect of PMA and Me_2SO on incorporation of radioactivity into intracellular protein and protein secreted by hepatocytes

Treatment*	Time in culture, days†	Incorporation into intracellular protein: cpm × 10^{-6}/culture	Incorporation into intracellular protein: % of control	Incorporation into secreted protein: cpm × 10^{-6}/culture	Incorporation into secreted protein: % of control
No drug	28	2.03	100	0.393	100
PMA, 10 ng/ml	28	1.81	89	0.339	86
PMA, 20 ng/ml	28	1.86	92	0.165	42
PMA, 100 ng/ml	28	2.48	122	0.281	72
Me_2SO, 2%	28	7.52	370	1.01	257
Me_2SO, 2%	43‡	5.67		1.24	

*Medium containing PMA or Me_2SO was used to feed cells from day 1 after plating until time of harvest (day 28 or day 43).

†Days after plating. Cells were radioactively labeled for 6 hr at this time and the medium and cells were harvested.

‡No PMA or control cultures were labeled on day 43 because no viable cells remained in the cultures at this time.

cells from a rat liver epithelial cell line. In other reports and from our own experience, comparable parenchymal cell morphology or the characteristic cordlike orientation has not been seen in hepatocyte cultures for more than a few days in the absence of cocultivation. The experiments reported here were terminated at a maximum of 43 days; however, in ongoing studies, cultures at 70 days have not begun to deteriorate. Hence, we do not know the maximum length of time hepatocytes can be maintained *in vitro* on medium supplemented with Me_2SO.

The mechanism of action of Me_2SO on MEL cell differentiation is not established, but various theories have been proposed. Because Me_2SO penetrates biological membranes (36), the drug may function as a carrier for specific nutrients and hormones. Me_2SO also alters the structure of proteins and nucleic acids and, as such, may directly alter gene expression. In its ability to maintain differentiation of normal adult hepatocytes, Me_2SO may act directly by controlling expression of differentiated liver-specific gene products or indirectly by altering hepatocyte responsiveness to other components, such as hormones or nutrients. The system described here uses chemically defined medium and is ideal for testing whether Me_2SO acts by altering hepatocyte responsiveness to medium components.

Me_2SO-treated hepatocytes may be irreversibly on their way to cell death even though death has been delayed as long as 70 days. Alternatively, the cells may retain the ability to resume proliferation. A way to test this possibility would be to add a mitogen such as EGF to Me_2SO-treated cells. EGF was not present in HCD medium; however, EGF was present in the medium used by other investigators to show that hepatocytes plated at low density can proliferate (15). Recent studies using intact normal liver have shown that addition of Me_2SO stimulates tyrosine residue phosphorylation of the EGF receptor (37). Questions that need to be addressed include the following: (*i*) What effect does Me_2SO treatment or release from the drug have on EGF receptors of hepatocytes? (*ii*) Do EGF or other mitogens stimulate DNA synthesis and subsequent cell division in Me_2SO-treated hepatocytes? (*iii*) What effect does addition of EGF have on expression of differentiated functions and morphology? (*iv*) Is the length of time cells are treated with Me_2SO critical to whether proliferation can be triggered?

Cultures containing Me_2SO undergo a variety of changes from time of plating until day 20. From day 20 until at least day 43, the cultures undergo little change except for a low level of cell division and cell death and, as such, a plateau in which the state of differentiation is stabilized can be generated. Not only differentiation but also cell number is retained; this will permit biochemical and molecular studies that cannot be carried out when large numbers of cells in the monolayer die after short periods of time and are not replaced. Further biochemical analysis is required to determine the normality of these hepatocytes with regard to other liver-specific functions, such as hormonal induction of specific enzymes, maintenance of cytochrome P-450, and ability to activate chemical carcinogens. The abnormal mitochondria and filament bundles present in some cells are a reminder that a drug was used to induce differentiation, a fact that has to be taken into consideration in interpreting the results of experiments. Nevertheless, a system for long-term culture of hepatocytes is needed for numerous studies, including those involving virus infection and transformation. This system, although not completely normal, opens many possibilities for investigating differentiated liver cells.

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