Culture of Human Fetal Liver[1] (37565)

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In vitro cultivation of differentiated human parenchymal liver cells has proved to be of some difficulty (1), although, short-term growth and maintenance of parenchymal-like cells from human fetal liver has been reported by a number of workers. Hillis and Bang (2) using tissue explants on a collagen substrate and a complex media, maintained human fetal parenchymal liver cells *in vitro* for periods of up to 100 days. Festenstein (3) maintained the outgrowth of cells from human fetal liver fragments on collagen-coated polyethylene discs for 39 days. Zuckerman, Tsiquaye and Fulton (4) maintained human fetal parenchymal hepatic cells for 10 to 16 days by pressure-implanting trypsinized suspensions onto polyethylene discs coated with mouse plasma. McLimans (5) dissociated human fetal liver by incubating and mincing in a salt solution of high osmolarity followed by repeated pipetting. The cells were grown out on a collagen substrate and parenchymal cells were maintained for up to 5 mo in steady-state rocker cultures. Bissell and Tilles (6) using trypsin-dispersed tissue, maintained strands of human fetal parenchymal liver cells for up to 4 wk. The present report describes a facile method for the preparation of collagen–substrate cultures of human fetal liver and the growth and long-term maintenance of differentiated hepatic parenchymal cells in these cultures. Maintenance of human fetal liver in Gelfoam-sponge organ cultures is also described.

Materials and Methods. Human fetal liver was obtained from fetuses obtained by hysterotomy. Cultures were prepared from 16 specimens. The fetal ages varied between about 8 and 18 wk and crown to rump measurements from 4 to 14 cm. The liver was removed and placed immediately in 10 ml of McCoy's 5a (Microbiological Associates, Bethesda, MD) containing 10% fetal bovine serum in a 125 ml flask at 4° for transportation to the laboratory. Elapsed time before receipt of the liver was 1 to 2 hr. The liver tissue was gently pressed through a 40 mesh (40 strands/in.) stainless steel wire screen into 5 ml of Hanks' balanced salt solution (BSS) using the plunger of a plastic 10 ml syringe. The liver fragments, which measured 0.3 to 0.5 mm, were washed twice by settling in BSS in a 15 ml centrifuge tube and suspended in media consisting of either RPMI 1629 or McCoy's 5a (Microbiological Associates) containing 10% fetal bovine serum and 100 units of penicillin and 100 μg of streptomycin/ml. One-tenth milliliter of tissue fragments in 2 ml of media was added to T-30 flasks (Bellco, Vineland, NJ) containing 2 to 4 glass coverslips coated with bovine collagen and the flasks gassed with 5% CO_2 in air. The collagen solution was prepared from lyophilized bovine Achilles tendon (Schwarz/Mann, Orangeburg, NJ) as follows: 300 mg of lyophilized tendon was cut with a scalpel into fine fragments and soaked overnight in 50 ml of 0.15 *M* NaCl. The saline was replaced with 1:1000 acetic acid and the fragments were homogenized at top speed for 2 min in a Virtis homogenizer at 4°. The T-flask surface and coverslips were coated with collagen by distributing 0.8 ml of the collagen solution over the surface and then heating the flask at 160° for 15 min. This method deposited a thin, stable film of denatured collagen which microscopically had a finely fibrous structure. The method of heat-treatment for the preparation of a collagen film on glass has previous-

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ly been described by McLimans (5). After addition of the liver fragments in 2 ml of media, the flasks were incubated overnight in a horizontal position to allow attachment of the fragments. Two additional milliliters of media were then added and the flasks were placed on a rocking apparatus as described by McLimans *et al.* (7). In the rocking apparatus, the flasks were held at an angle of 25° from the horizontal with the media collected in the bottom 2 cm of the flask. Every 3 min the flasks were automatically rocked through 50°, thus maintaining a thin film of media over the tissue fragments. Alternatively, the flasks were attached by a simple spring clamp to a roller drum and rotated at 14 rev/hr (Wheaton Vitroller, Millville, NJ). The incubation temperature was 37°. Culture media was generally changed twice weekly.

For microscopic observation the coverslips were fixed in Bouin's solution containing 5% acetic acid and stained with hematoxylin and eosin. Glycogen was demonstrated by fixation in Bouin's or absolute ethanol and staining by the periodic acid–Schiff method as described by Pearse (8), after McManus. Diastase-pretreated preparations served as controls.

Sponge-organ cultures were prepared by placing 0.05 ml of the liver tissue fragments on pieces of Gelfoam sponge (Upjohn, Kalamazoo, MI) measuring 12 × 6 × 3 mm previously soaked in BSS. The sponge cultures were maintained in T-flasks on a rocker apparatus. For microscopic observation the sponge cultures were fixed in Bouin's, paraffin embedded and sectioned at 5 μm.

Results and Discussion. The method for preparation of the collagen substrate is less complex than that usually employed (9) and produced a stable, finely fibrous structure that supported growth and maintenance of parenchymal cells from the liver fragments. Poor cellular outgrowth from the liver fragments was observed in the absence of a collagen substrate.

The method used to disperse the liver tissue into 0.3 to 0.5 mm fragments is simple and also efficient in that a large majority of the tissue fragments attached to the collagen substrate after overnight incubation in the horizontal position. An outgrowth of cells was observed from most of the attached tissue fragments after 3 to 5 days incubation. Use of the rocker apparatus or the roller drum gave essentially comparable results. Microscopically, the cellular outgrowth was made up of sheets and cords of cells with a regularly polygonal structure and a very granular cytoplasm in the unstained state and containing a spherical nucleus with 1 or 2 spherical nucleoli [Sandstrom's type IV cell (10)]. The outgrowing cells displayed the typical morphology and arrangement of differentiated hepatic parenchymal cells (hepatocytes) as observed in sections of human fetal liver fixed on receipt. The area of hepatocyte outgrowth continued to increase for 10 to 14 days and mitotic figures were readily observed in the cells during this period. Figure 1A illustrates the appearance of the outgrowth of hepatocytes from a liver explant of a 15 wk fetus, after 21 days of culture. Figure 1B is a higher magnification of an area of the same culture. Note the polygonal structure of the cells and the spherical nuclei containing 1 or 2 spherical nucleoli. Areas of formation of cells in the granulocytic and erythrocytic series were prominent in the first 2 wk of culture and were largely confined to the explanted tissue. An area of formation of cells in the granulocytic series is illustrated by Fig. 1C. Glycogen was readily demonstrated in the cultured hepatocytes and Fig. 1D illustrates glycogen formation after 36 days of culture, as demonstrated by the PAS reaction.

Cultures were prepared from 16 specimens with a gestational age between about 8 and 18 wk. A satisfactory outgrowth of hepatocytes was obtained with liver fragments from fetuses of 12 to 18 wk gestation and the cells could be maintained for periods of at least 60 days. Cultures prepared from the smallest fetus, of about 8 wk gestation (4 cm) did not produce as large an outgrowth of hepatic parenchymal cells and these cells degenerated within 2 wk.

Fibroblastic growth surrounding the colonies of hepatocytes was observed after about 1 wk of cultivation. This growth became excessive and intruded on the hepatocyte colonies after about 3 wk of culture. The addi-

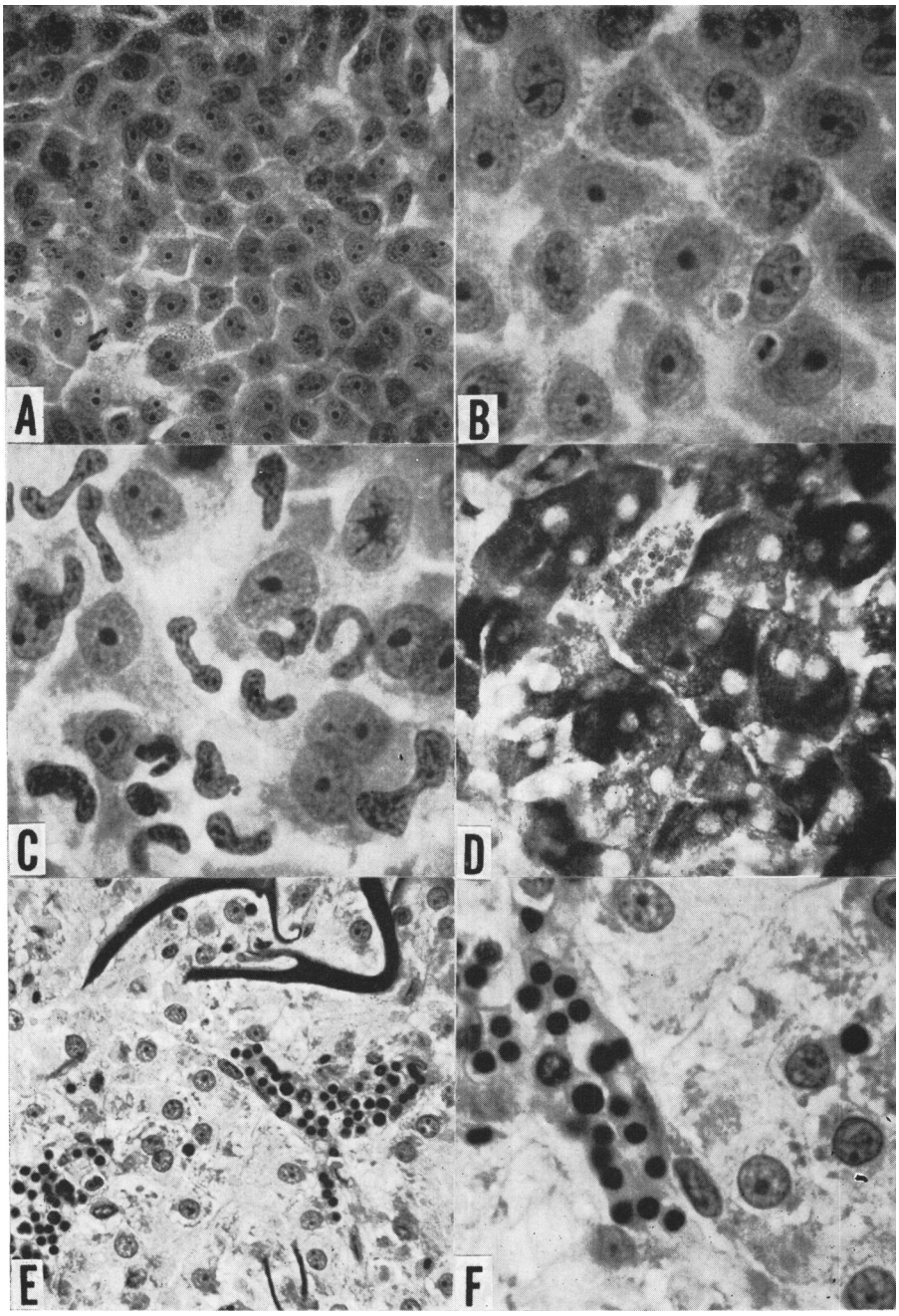

FIG. 1A. Outgrowth of hepatocytes from liver explant of 15 wk human fetus at 21 days. H & E 213×. (B) Outgrowth of hepatocytes, higher magnification. 536×. (C) Area of formation of cells in granulocytic series, 21 days. H & E 536×. (D) Glycogen formation by cultured hepatocytes at 36 days. PAS 213×. (E) Sponge organ culture of human fetal liver at 8 days, showing area of developing erythroblasts. Paraffin section H & E 213×. (F) Sponge organ culture of human fetal liver 8 days, at higher magnification. H & E 536×.

tion of 20 μg/ml of hydrocortisone (Merck, West Point, PA) to the media resulted in a marked curtailment of the fibroblastic growth. In the presence of hydrocortisone, fibroblastic growth was limited to areas exterior to the hepatocyte colonies even after 6 wk of culture.

The morphology of the fetal hepatic parenchymal cells was particularly well-maintained in the Gelfoam-sponge organ cultures and compared favorably with parenchymal cells in fetal liver fixed immediately upon receipt. Figure E illustrates a sponge organ culture of human fetal liver after 8 days of culture, fixed in Bouin's, sectioned at 5 μm and stained with hematoxylin and eosin. Note the 2 foci of erythroblasts in various stages of development and the spicules of Gelfoam sponge at the top of the illustration. Figure 1F, a higher magnification, illustrates in greater detail the hepatic parenchymal cells and the foci of erythroblasts. Note that the membranes of adjacent hepatocytes are closely adherent and that the vesicular spherical nuclei contain 1 or 2 spherical nucleoli. The cell with an elongated nucleus adjacent to the foci of erythroblasts in Fig. 1F is probably a supporting connective tissue cell.

Summary. A method for the preparation of collagen–substrate cultures of human fetal liver is described. The growth and long-term maintenance of differentiated hepatic parenchymal cells in these cultures from liver tissue from human fetuses of 8 to 18 wk gestation is described. Satisfactory outgrowth of hepatic parenchymal cells was obtained from fetuses of 12 to 18 wk gestation, while outgrowth of cells from an 8 wk fetus was less satisfactory and the cells degenerated within 2 wk. Outgrowing cells demonstrated the typical morphology and arrangement of hepatic parenchymal cells and glycogen formation was readily demonstrated. Mitotic figures were observed during the first 2 wk of culture and foci of cells in the granulocytic and erythrocytic series were prominent during this period. Addition of hydrocortisone to the media resulted in a marked reduction of fibroblastic growth surrounding the colonies of hepatic parenchymal cells, even after prolonged culture. Culture of human fetal liver in Gelfoam-sponge organ cultures is described. In these organ cultures the morphology of the hepatic parenchymal cells compared favorably with the parenchymal cells in fetal liver fixed immediately on receipt. A simple method for preparation of the collagen substrate is described.

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