MORPHOLOGY AND FUNCTION OF CELLS OF HUMAN EMBRYONIC LIVER IN MONOLAYER CULTURE

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ABSTRACT

A system for culturing human fetal liver cells in monolayers is described and the effects of various conditions of growth on the morphology and function of the cultured cells are presented. The addition of 10% calf serum or 1% human serum to the growth medium accelerated the proliferation of the liver cells, with subsequent loss of characteristic morphology and specific functional activity. In the absence of serum, the cultured liver cells retained their morphology and their function for at least 4 wk, as evidenced by secretion of serum albumin and storage of glycogen and iron.

INTRODUCTION

Successful cultivation of human liver for more than a few days has generally required organ culture techniques, in which peripheral outgrowth of cells occurs around small liver explants placed on a variety of physical supports (1, 2, 3). Cultivation of hepatoma cells from the rat has been described (4). In addition, rat liver cells have been grown by cloning from single cells, but the detailed methodology has not been reported (5).

The present report describes a system for the culture of human embryonic liver cells that utilizes standard monolayer techniques. The success of the cultures and the effect of various manipulations were demonstrated by cellular morphology, histochemical identification of intracellular substances, and by assay of serum albumin secreted into the culture medium.

METHODS

Tissue

The human liver was obtained from 10–19 wk embryos, delivered by abdominal hysterotomy. The whole liver was placed in phosphate-buffered saline (PBS) (supplemented with potassium penicillin, G

150 μg/ml, and streptomycin sulfate, 250 μg/ml, but free of calcium and magnesium) and promptly stored at 4°C.

Medium

The standard medium was that of Leibovitz ("L-15", Microbiological Associates Inc., Bethesda, Md.), supplemented with 0.042% arginine (HCl), 0.03% glutamine (Microbiological Associates Inc.), 0.1% dextrose and antibiotics, as above. Further supplementation with fetal calf serum (FCS, Flow Laboratories, Inglewood, Calif.), human serum, bovine serum albumin (Armour Pharmaceutical Co., Kankakee, Ill.), human serum albumin (Sigma Chemical Co., St. Louis, Mo.), or Ficoll (Pharmacia Fine Chemicals Inc., Piscataway, N. J.) was evaluated. Eagle's minimal medium (Grand Island Biological Co., Berkeley, Calif.) with Earle's salt solution supplemented with antibiotics, sufficient sodium bicarbonate to give a pH of 7.3, and 10% fetal calf serum (FCS) was also tested.

Trypsinization

Trypsin concentrate was prepared and stored according to Wallis (6) and used as a fresh 0.1%

working solution with antibiotics added. Liver tissue was separated from the gall bladder and intrahepatic fibrous tissue by blunt dissection, minced, and washed into a 500 ml trypsinizing flask with 2–3 ml of PBS and then 25 ml of trypsin solution. The time elapsed between delivery of the fetus and the start of trypsinization was less than 1 hr.

Trypsinization proceeded at room temperature with magnetic stirring. After 15 min, the undigested tissue was allowed to settle and the initial fraction of suspended cells was decanted into a centrifuge tube containing 5 ml of FCS. A second 25 ml of trypsin solution was added to the flask and trypsinization was carried out for another 30 min. The suspended cells were then harvested as before. Depending on the size of the specimen, two or three further 30-min cycles of trypsinization were carried out until essentially all of the original tissue had been digested. The tubes stood at room temperature for approximately 1 hr, which favored the accumulation of the large "liver cells" over the more slowly sedimenting hematopoietic elements. A red-brown pack formed at the bottom of the tubes. After the upper two thirds of the supernatant was decanted, the residual cells were centrifuged at 100 g for 5–10 min. The pellets were resuspended in fresh growth medium, washed three times, and resuspended to give a total cell concentration (exclusive of red cells) of 20 million/ml.

Cultivation

A suspension of 10 million cells/ml was inoculated in 2.0 ml portions into 35 mm tissue culture plates (Falcon Plastics, Division of B-D Laboratories, Inc., Los Angeles, Calif.) with and without sterile cover slips. In a separate study, a solution of rattail collagen was prepared according to Ehrmann and Gey (7), reconstituted by dialysis against distilled water, and applied to the sterile cover slips used. For some studies[1] an inoculum of 0.5 ml of the same suspension was placed in (stationary) constricted glass tissue culture tubes (Rochester Scientific Co., Rochester, N.Y.). In general, cells were allowed to adhere and proliferate for 2 days, when the initial medium was aspirated, the cells were washed, and fresh growth medium was applied. Acidity was accurately corrected to a maximum pH of 7.3 with bicarbonate buffer (4 parts L-15, one part 7.5% sodium bicarbonate) 0.02–05 ml/plate, as needed.

Stains

Cells on cover slips or plates were fixed in absolute methanol at various stages of growth and stained

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with hematoxylin and eosin, the periodic acid Schiff (PAS) stain or Gomori iron stain (8). The freshly trypsinized cells were examined by air-drying a drop of suspension on a glass slide and fixing in 95% ethanol.

Albumin Assay

The concentration of serum albumin in culture fluids was measured by a radio immunoassay described elsewhere[1].

Frozen-Stored Cells

Freshly trypsinized liver was suspended at a concentration of 20 million cells/ml in L-15 supplemented with 20% FCS. One ml portions of the suspension were mixed with 1 ml of L-15 supplemented with 20% dimethyl sulfoxide, and the mixtures were transferred without delay to 2.5-ml sterile vials. The vials were flame-sealed and placed in an ethanol-glycerin (70:30) bath, precooled to 4°C. The bath was then placed in a −70°C freezer. After 1 hr, the vials were removed from the bath for permanent storage at −70°C.

RESULTS

Fresh Cells: Inoculum

A typical preparation of trypsinized human embryonic liver contains both hematopoietic elements and large eosinophilic cells with small nuclei, in a ratio of approximately 100:1. The latter are presumed to be mature hepatic parenchymal cells although not necessarily the only type of liver cell present, since a hepatocyte precursor might well differ morphologically from the mature cell. The trypsinizing method produces about equal numbers of single cells and formations appearing as pairs, triplets, and occasional larger groups of hepatocytes, regardless of the duration of trypsinization (Fig. 1.). "Hepatocytes" in the fresh preparation stain variably with PAS, depending on the age of the embryo: those from the older embryos (12–14 cm) stain faintly, whereas those from the younger embryos (4–7 cm) do not stain at all. Hepatocytes from the latter contain no stainable iron, while those from older embryos may have a diffuse bluish hue with Gomori's method.

Early Growth Phase

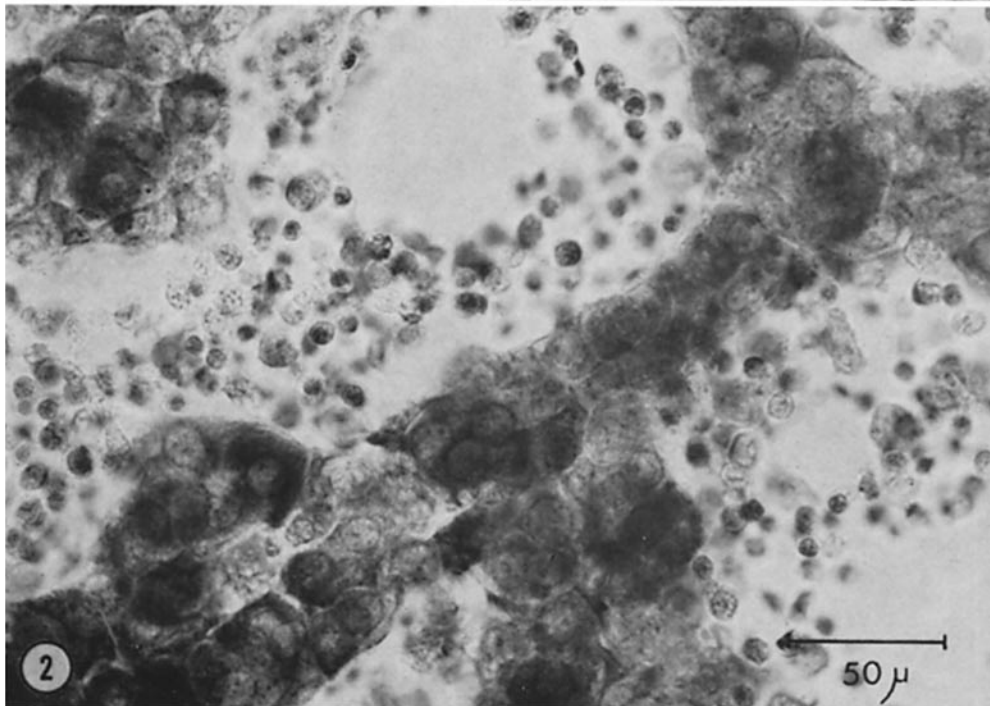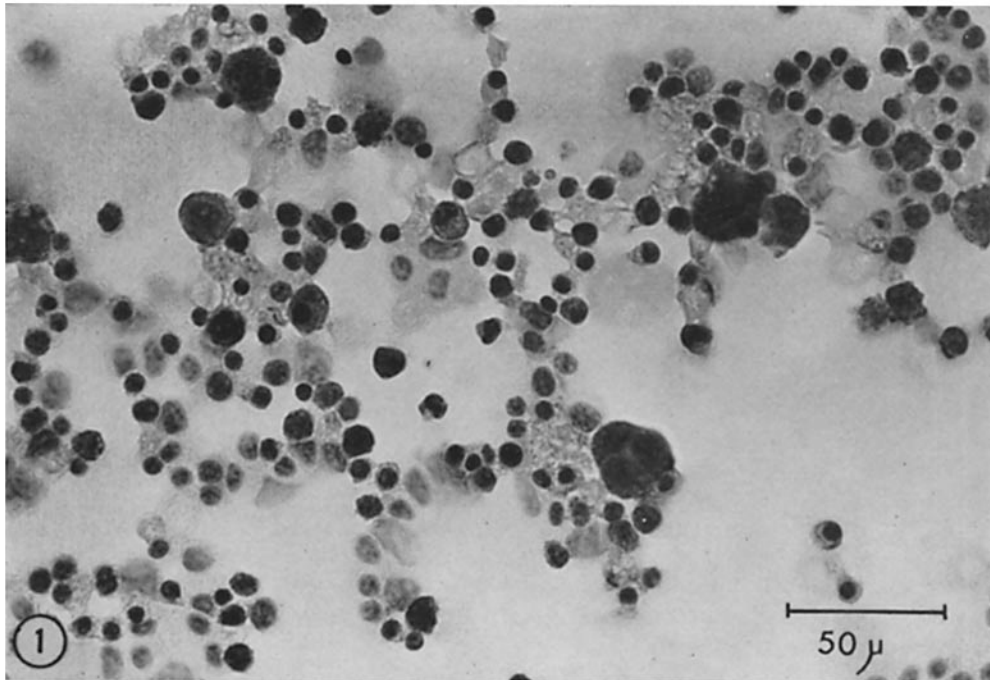It is clear from the 12-hr growth of the trypsinized preparation (Fig. 2) that a sizable proportion

FIGURE 1   Trypsinized preparation of embryonic liver. Hematoxylin and eosin. Large cells with dark cytoplasm are the presumed hepatocytes. × 400.

FIGURE 2   Human embryonic liver, 12 hr. PAS stain (dark material of cytoplasm). The liver cells have formed crude trabeculae encircling hematopoietic elements. × 400.

of cells presumed to be hepatocytes adhere to the dish or cover slip and, once established, undergo rapid hyperplasia. Over this same 12-hr period, virtually all of the attached and growing hepatocytes acquire a marked affinity for PAS stain, which would be consistent with the ability to synthesize and store glycogen. Gomori's stain also gives a positive reaction showing dense collections of blue granules, usually in a perinuclear array.

Cells rapidly acquired these functions with most of the media used, including serum-free medium, although cell outgrowth was accelerated in media that contained 10% FCS or 1% human serum (HS), the latter being superior in most cases. Dextrose was added routinely because attachment and early growth seemed depressed in its absence whether or not serum was added. Other factors of importance in the early phase of growth were the density of the cell inoculum and, to a lesser degree, the age of the embryo. The optimum inoculum was found to be 10 million total cells/ml. An inoculum of 20 million cells/ml, or more, usually resulted in poor attachment and growth of cells because of the adverse affects of overcrowding; 2.5 million cells/ml, or less, produced widely scattered colonies of liver cells that usually failed to thrive. In general, preparations from young embryos did less well, possibly because of a smaller proportion of liver cells in those specimens.

Late Growth Phase

Beginning on the third day, marked differences were found between serum-containing and serum-free cultures. The 3-day culture with FCS showed a dense growth of attenuated, large liver cells spreading in typical monolayer growth (Fig. 3); at 2 wk, there was still greater variation in the size and shape of cells and in the number and size of nuclei, and there was a moderate growth of spindle-shaped cells. A number of cells were difficult to classify; they were presumably of bile duct or endothelial origin, but they might have been altered liver cells. The PAS positivity of the culture as a whole was diminished at 3 days and nearly absent after 2 wk of growth. Stainable iron decreased in parallel until it was virtually absent at 2 wk.

The cells grown without serum, on the other hand, presented a striking contrast to the cultures just described. On the third day of cultivation, the density of growth was clearly less than that of the serum-exposed cells. There were signs of organized

growth which were not seen in cultures exposed to serum. Growth was restricted almost entirely to the formation of trabeculae, one or two cells thick, which extended from the many isolated colonies that formed after the initial division of the seeded cells (Fig. 4). Outgrowth of broad, attenuated cells, such as occurs in random manner in the serum-containing culture, was absent. Preservation of function is inferred from the strong PAS positivity of these cells. Gomori's iron stain was also diffusely positive. The 2-wk culture revealed further growth of the same character, with covering of the glass by a network of trabeculae, many several cells wide, and the formation of acinus-like structures (Figs. 5, 6). PAS positivity remained strong. Stainable iron was present although more diffuse and of lower intensity than at 3 days.

The function of cultures during the late growth phase was further investigated by serial determinations of human serum albumin in the culture fluid (Fig. 7). The presence of FCS in the medium resulted in an abrupt diminution in albumin secretion after 1 wk of cultivation. In the serum-free medium, on the other hand, secretion continued for 11 days and the absolute amount of albumin secreted was at least five times that in the serum-containing culture. The cultures were followed for 18 days, the medium being changed only to the extent of replacing each 0.2 ml withdrawn for assay of albumin. With complete change of the medium on day 18, reassay on day 21 and beyond showed that the serum-free culture was still capable of producing albumin at the same rate as before (Fig. 7). Albumin secretion beyond day 21 was also observed in the culture with 10% FCS, although at a markedly lower level. The serum-free culture also secreted larger amounts of albumin when the medium was changed daily (Fig. 8). In addition, the frequent medium changes appeared to have no deleterious effect on the morphology of the culture. Thus, serum-free medium favored the secretion of albumin as well as the presence of glycogen and the secondary organization of cells.

Cells grown in medium which was supplemented for the initial 48 hr with human serum, 1%, and then was subsequently devoid of serum provided a monolayer in which the hepatocyte glycogen was apparent for slightly longer periods than in the monolayers grown with the continual presence of 10% FCS. In some cases secondary and tertiary
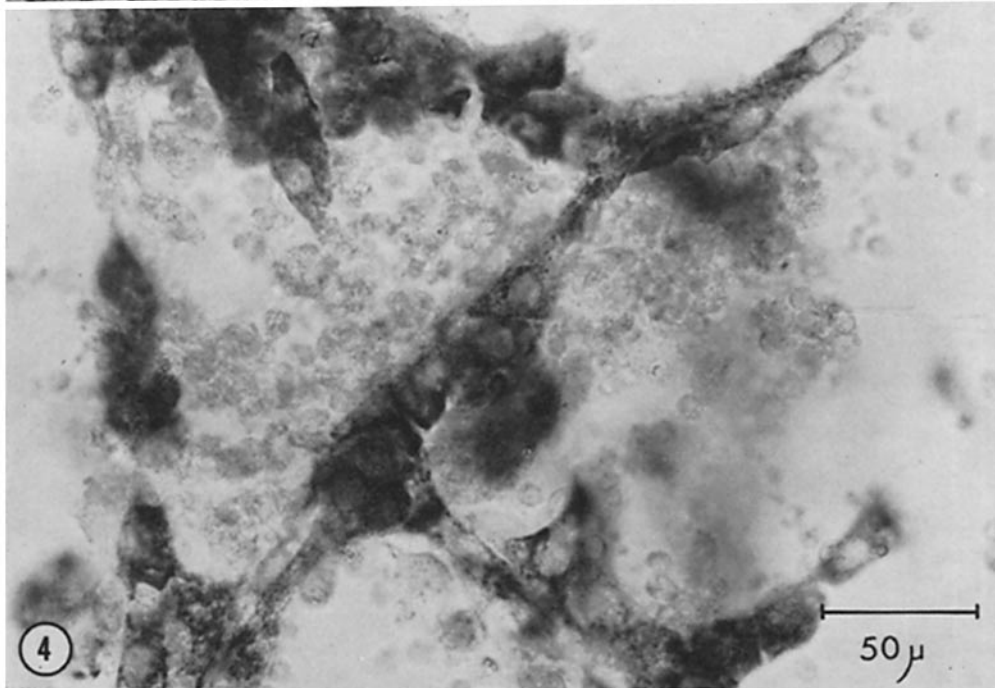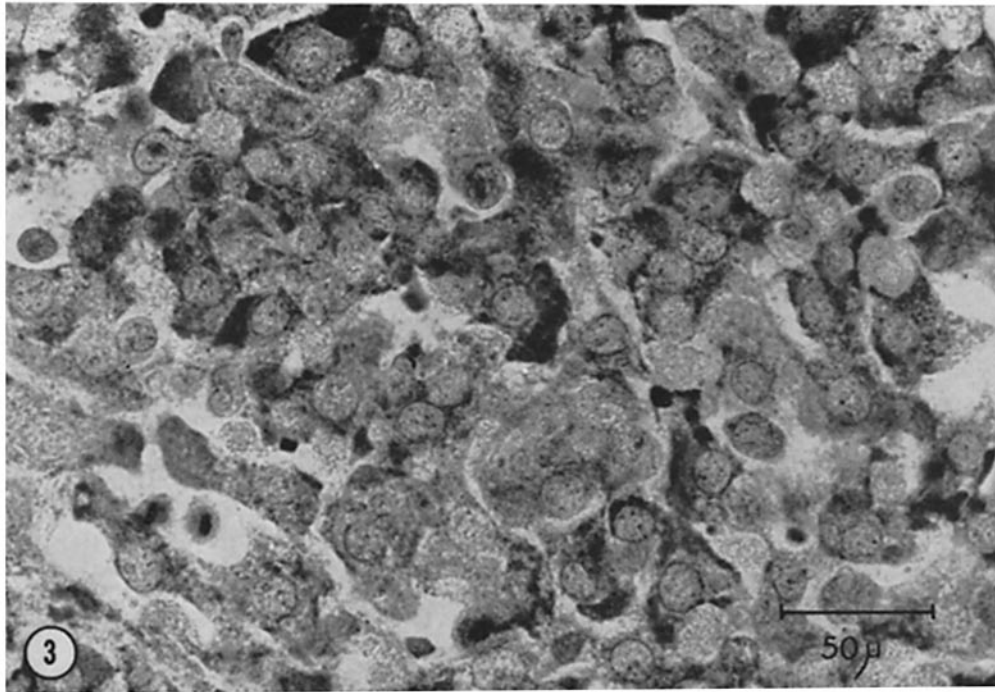
FIGURE 3   Human embryonic liver grown in medium supplemented with 10% fetal calf serum, day 3. PAS. × 400.

FIGURE 4   Human embryonic liver grown in serum-free medium, day 3. PAS with no counterstain (hazy material is surrounding hematopoietic elements or PAS-negative liver cells). × 400.
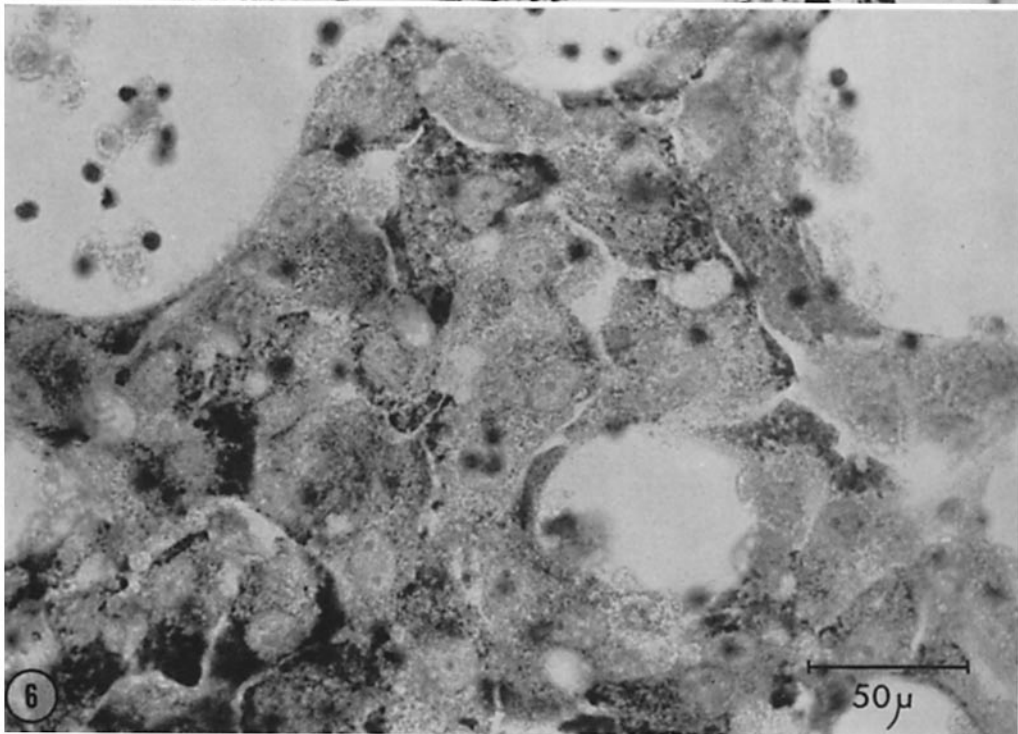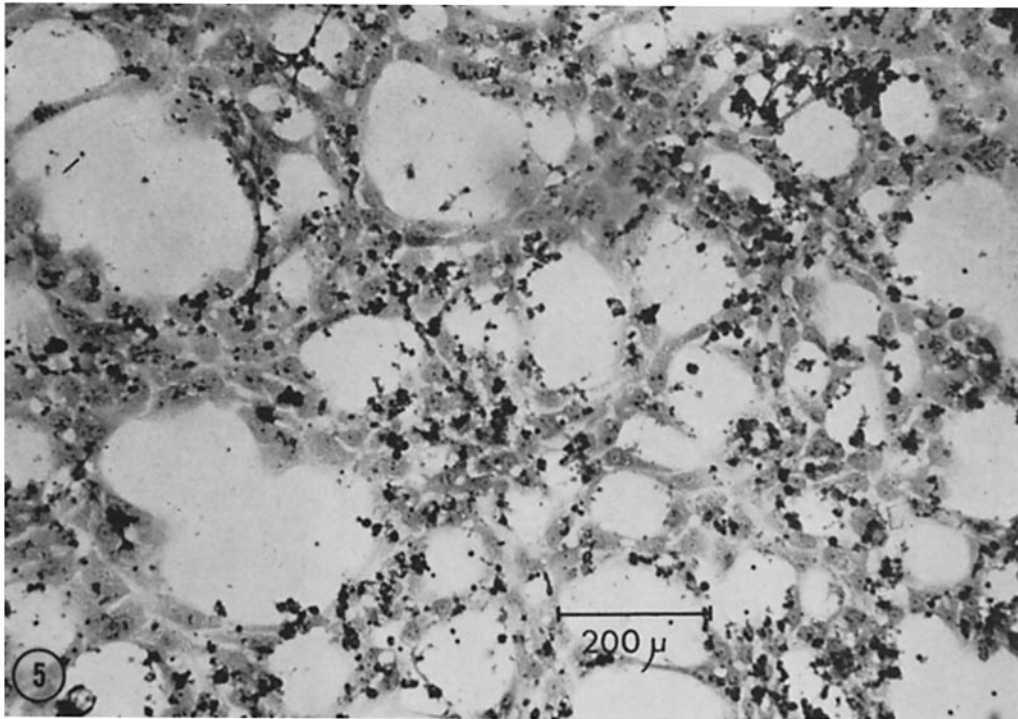
FIGURE 5   Human embryonic liver grown in serum-free medium, day 14. Hematoxylin and eosin. × 90.

FIGURE 6   Same as Fig. 5. PAS stain. Aciniform structures of varying size are present in the culture. × 400.
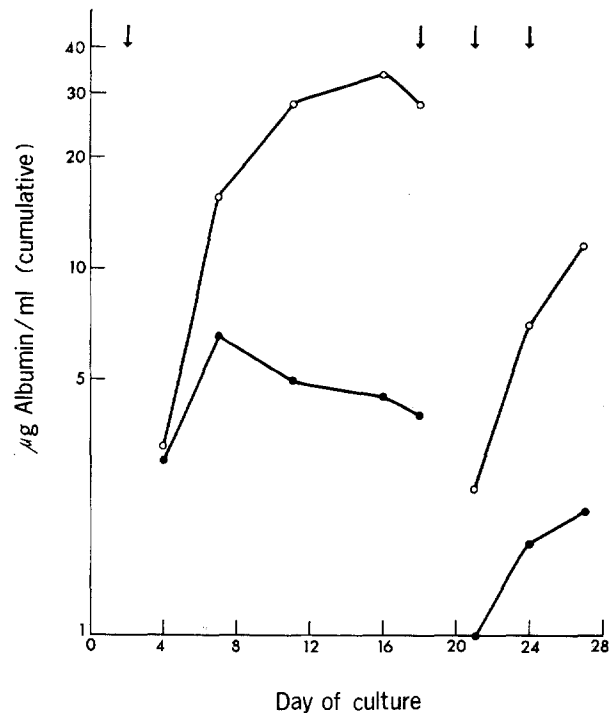
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FIGURE 7 Albumin secretion: infrequent change of medium. Albumin secretion by cultures grown in medium with 10% FCS, ●, or devoid of serum, O, and medium changed infrequently. Each point represents withdrawal of 0.2 ml of medium for assay of albumin, with the same volume of fresh medium returned to the culture. A correction for the resulting dilutions has been made in plotting the points. Arrow indicates change of medium in both cultures.

growth occurred as in the serum-free culture. With different human sera, however, the outcome was variable, and with some sera the 2-wk cultures were nearly as lacking in secondary structure as the calf serum cultures of the same age.

Eagle's medium was found to give satisfactory initial outgrowth of liver cells if the pH was maintained by careful control of both bicarbonate concentration and pCO_2. Even with carefully regulated pH, however, late growth of the culture was less satisfactory in Eagle's than in L-15.

Ficoll, 0.5–3.0% in L-15, was tested as a non-protein macromolecule that might promote attachment and early growth of cells by providing the physical attributes of serum macromolecules without the metabolic effects of serum. The cultures grown in serum-free medium, however, were not noticeably benefitted by the presence of Ficoll. A similar result was obtained in serum-free cultures supplemented with either bovine or human serum albumin, 0.4%.

The addition of rattail collagen was found to promote cell outgrowth, but the cultures failed to achieve any degree of secondary organization and were short-lived.

The initial outgrowth of cell cultures grown in glass tubes paralleled that described above for cultures grown in plastic tissue culture dishes (with or without glass cover slips). However, in tube monolayers containing serum, a typical "fibroblast" overgrowth was apparent after 1 wk, and a rapid and almost complete replacement of the liver cells occurred over the succeeding few days. In serum-free tube cultures, fibroblast overgrowth was not a problem, but extensive trabecula formation was seen only rarely. The hepatocytes, instead, had a tendency to remain in separate islands of densely packed cells. Such cultures appeared viable and produced albumin for a period of about 2 wk. The rolling of tube cultures (15 rev/hr) did not increase the survival of liver cells or promote greater production of
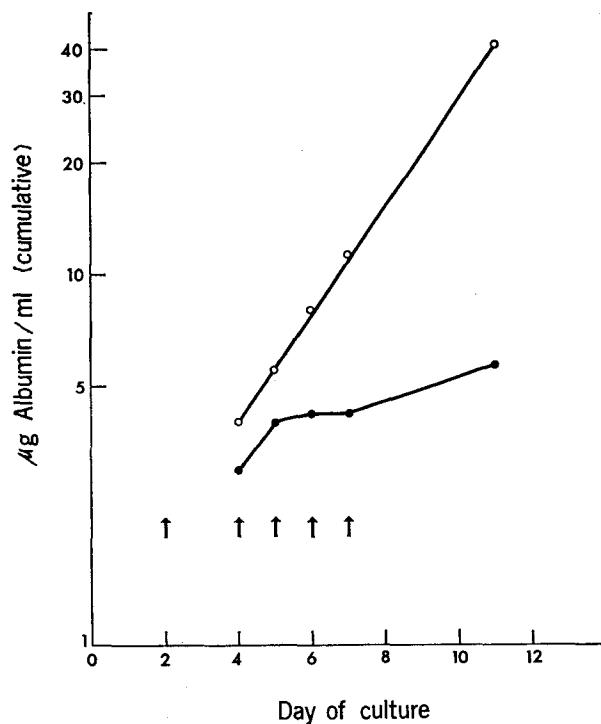
FIGURE 8 Albumin secretion: frequent change of medium. Albumin secretion by cultures grown in medium with 10% FCS, ●, or devoid of serum, O, and medium changed frequently. The cultures were grown parallel to those of Fig. 7, and were from the same embryo (see Methods). Each point represents the cumulative secretion of albumin. Arrow indicates change of medium in both cultures.

albumin as compared with parallel cultures in stationary tubes.

Frozen-Stored Cells

Frozen cells were thawed, washed three times in growth medium, resuspended, and inoculated in the standard volumes and concentration (see Methods). At 24 hr, small colonies of cells were present as with freshly trypsinized cells, and, by 48 hr, there was considerable cell outgrowth. It was relatively more difficult, however, to establish a culture with frozen-stored cells than with freshly trypsinized tissue. Therefore, for consistent results, the medium was routinely supplemented with serum, and an outgrowth typical of serum-exposed cells was seen. As with fresh cells, more prolific outgrowth was achieved with 1% HS or 10% FCS. The rate of albumin synthesis of such cultures was 25–50% that of the parent preparation, which may represent, functionally, the plating efficiency of these cells.

DISCUSSION

The present work was undertaken in an effort to establish a dependable system for monolayer cultivation of fetal liver cells. It became necessary, first, to characterize the morphology and function of liver parenchymal cells in tissue culture. The criteria derived for liver cell identity and function were then utilized to evaluate the relative efficacy of various growth conditions.

Morphologically, the liver cells were found to be large and cuboidal and to have relatively small nuclei and prominent nucleoli. With advanced growth, under optimal conditions, the cells retained the same physical features but secondarily formed cords and acinus-like structures (Fig. 5). There was also evidence of abortive tertiary growth, seen microscopically as heaped-up and probably degenerating cells, and grossly as a stippled pattern of the culture surface.

Liver cell function was demonstrated both by the quantitation of albumin released into the

medium and by the demonstration, histochemically, of the presence of glycogen and iron within the cells. Secretion of albumin appears to be specific for liver parenchymal cells in culture[1]. Although the PAS reaction is positive for the 1, 2 glycol linkage of many other carbohydrates as well as glycogen, it is considered most likely that the diffuse, intracellular, reacting substance in these cells is, in fact, glycogen (9). Although glycogen occurs in cells of various organs in vivo, the presence of glycogen in these cultures parallels the other parameters measured and would appear to be a reliable way to distinguish liver parenchymal cells from other cell elements. The presence of stainable iron was a third parameter examined. The ability to store iron is recognized as a property of normal liver, although the processes involved remain obscure. It is thought that liver parenchymal iron does not readily, if at all, exchange with plasma iron in man (10) and may not be released except with destruction of hepatocytes, as in hepatitis (11). The supply of iron in the present cultures may be generous, since hematopoietic elements are numerous. The stainable iron of the liver cells, however, declines in parallel with loss of other liver cell functions, even without changes in medium. This simple histologic method may be another useful way in which to examine the stability and function of liver tissue in vitro.

To evaluate the efficacy of growth conditions for monolayer cultivation, it became useful to distinguish between the rapid growth of the first 48 hr (early phase) and the more stationary condition of the subsequent several weeks (late phase). It was found that supplementation of the medium with calf or human serum led to rapid proliferation in the early phase, but this proliferation was clearly associated with attenuation of primary and secondary morphology and early loss of function in the late phase. Conversely, serum-free medium yielded relatively slow early phase growth, although there was subsequent development of secondary morphology and a prolongation of full liver cell function for at least 4 wk.

Many of the difficulties described by others were met in the serum-containing cultures. Acid production was high, requiring daily addition of small amounts of buffer for several days. In a serum-free culture, on the other hand, acid production was so slight that buffer supplements were not usually necessary. Fibroblast growth in serum-free cultures

was minimal, although large spindle-shaped, PAS-negative cells appeared in areas peripheral to the growth of liver cells after 3 wk or more of culture.

The ability to grow without added serum may be unique to embryonic liver, although this has not yet been established. It may be only the liver that can provide, unaided, the proper conditions for attachment and growth, possibly by producing serum albumin and other extracellular substances[1].

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