Spheroidal Aggregate Culture of Rat Liver Cells: Histotypic Reorganization, Biomatrix Deposition, and Maintenance of Functional Activities

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ABSTRACT Liver cells isolated from newborn rats and seeded on a non-adherent plastic substratum were found to spontaneously re-aggregate and to form, within a few days, spheroidal aggregates that eventually reached a plateaued diameter of 150–175 μm. Analyses on frozen sections from these spheroids by immunofluorescence microscopy using antibodies to various cytoskeletal elements and extracellular matrix components revealed a sorting out and a histotypic reorganization of three major cell types. A first type consisted of cells that segragated out on the aggregate surface forming a monolayer cell lining; a second type was identified as hepatocytes that regrouped in small islands often defining a central lumen; and a third group of cells reorganized into bileduct-like structures. This intercellular organization in the aggregates was paralleled by the accumulation of extracellular matrix components (laminin, fibronectin, and collagen) and their deposition following a specific pattern around each cell population structure. Determinations of albumin secretion and tyrosine aminotransferase induction by dexamethasone and glucagon at various times after the initiation of the cultures revealed a maintenance of the hepatocyte-differentiated functions for at least up to 2 mo at the levels measured at 3–5 d. It is concluded that cells dispersed as single cells from newborn rat liver conserve in part the necessary information to reconstruct a proper three-dimensional cyto-architecture and that the microenvironment so generated most likely represents a basic requirement for the optimal functioning of these differentiated cells.

There have been long-recognized difficulties in defining optimal culture conditions for the maintenance of survival and functional activities by hepatocytes or by other differentiated epithelial cell types freshly dissociated from their in vivo environment; these difficulties probably reflect the complex nature of the regulatory mechanisms which operate in vivo and control growth and differentiation. Recent progress in tissue culture research has defined three major classes of regulatory factors: soluble factors which include nutrients, trace elements, hormones, and growth factors (1–4); extracellular matrix or substratum which permits not only proper cell attachment and shape but also may provide direct regulatory signals to cells (3–8); and, homologous and heterologous cell–cell interactions which somehow can regulate growth as well as functional activities (9–15).

One potentially limiting factor in attempting to artificially reproduce in monolayer cultures the various aspects of normal cell regulation is that under these culture conditions, in contrast to the in vivo situation, the cells are imposed a two-dimensional environment that may hamper some normal regulatory processes (16). Indeed, a number of studies making use of the three-dimensional re-aggregate (17) or spheroid (18) culture system have suggested that cells may require a proper three-dimensional cyto-architecture as found in vivo for optimal functioning (reviewed in 19). Cells dissociated from a variety of tissues, mostly of embryonic origin, have been demonstrated to be capable under appropriate experimental conditions to re-assemble into aggregates resembling the organization and architecture of their tissue of origin (17, 19–24). In such structures where no particular geometry is imposed to the cells and where cell–cell contacts are maximized, the cells survived longer while maintaining their differentiated functions and, often, continuing their normal differentiation (19, 24–27).

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In the present study, we show that rat liver cells, set up in primary culture under conditions in which attachment to substratum is prevented, can re-aggregate into small spheroids wherein they organize into histotypic ductular and hepatocytic structures and produce insoluble matrix materials that accumulate in specific patterns. Under these conditions, cell survival and differentiated functions are maintained for an extended period of time, even in the absence of added hormones or serum factors, suggesting that this particular in vivo–like microenvironment regenerated by the liver cells in the aggregates may provide the minimal essential requirements for the long-term maintenance of hepatocytes.

MATERIALS AND METHODS

Cell Isolation: Liver cells were isolated from 7–10-d-old Fisher 344 rats by an adaptation (28) of the two-step perfusion method (29). Briefly, the animals were anesthetized under ether and the abdominal cavity was opened to cannulate the vena porta and to section the vena cava. The liver was first washed with Ca^{2+}-free HEPES buffer (6.7 mM KCl, 142 mM NaCl, 10 mM HEPES, 0.45 mM NaOH, pH 7.4) at 37°C and then perfused with the same buffer supplemented with 0.05% collagenase and 5 mM Ca^{2+}. Finally, the liver was removed and the cell suspension made in Williams' medium E (Gibco Laboratories, Grand Island, NY). Except when specifically noted, no supplement was added to the basal medium.

Initiation of the Spheroid Culture: Spheroid cultures were initiated by seeding a total of 1×10^6 cells in 4 ml of medium per 60-mm petri dish precoated with poly (2-hydroxyethyl methacrylate) (poly-HEMA).[1] Coating with poly-HEMA was achieved by evaporating in each dish 2 ml of 95% ethanol containing 2.5% poly-HEMA (8). This provided a coat of sufficient homogeneity to completely inhibit cell attachment. The cultures were kept at 37°C in a humidified CO_2 incubator with weekly change of 50% of the medium.

Size and Multiplicity of the Aggregates: The spheroids were sized directly in the dishes using a microscope equipped with a graduated eyepiece or on photographs using a calibrated ruler. A spheroid volume (V) was calculated from measurements of 2 orthogonal diameters (a and b) using $V = \pi a^2 b/6$, in which $b > a$. The average diameter at one time point was then deduced from the geometric mean of volume determinations from 30 to 50 different spheroids. The number of cells per aggregate (multiplicity) was estimated using an electronic particle counter (Coulter ZBI) as the ratio of the number of particles (cells) at time zero to the number at a specified time. Details of the procedure to distinguish between loosely aggregated and tightly attached cells are given in the legend to Fig. 2.

Spheroid Histology: Cell identification in spheroids was achieved on the basis of morphological criteria on hematoxylin-eosin–stained sections of spheroids and through the use of immunofluorescence microscopy and antibodies against specific elements of the cytoskeleton. The preparation and characterization of the antibodies used in this study were reported elsewhere[2] (30, 31). VmAb is a mouse monoclonal IgG antibody that reacts specifically with vimentin and stained exclusively the nonparenchymal cells of the liver including fibroblasts, mesothelial cells, endothelial cells, and Küpffer cells. Anti-CK55 is also a mouse monoclonal IgG antibody. It reacts with a 55,000-dalton cytokeratin (CK55) and stained hepatocytes and other liver epithelial cells. Anti-CK52 is a polyclonal antibody prepared in the guinea pig. It reacts with a 52,000-dalton cytokeratin filament protein (CK52), and stained bile ductular cells, but not hepatocytes. In addition, three rabbit polyclonal antibodies were used to identify and locate extracellular matrix materials in the spheroids. Anti-fibronectin and anti-collagen (rat tail) were prepared as described (32); anti-laminin was purchased from Bethesda Research Laboratories, Gaithersburg, MD. Hematoxylin–eosin staining was performed on section of paraffin-embedded spheroids fixed in Bouin's for 2 h. For indirect immunofluorescence analysis, the aggregates from 3–10 dishes were pelletted, frozen in liquid nitrogen, and then processed for cryostat sectioning. Frozen sections were placed on microscope slides and air dried. They were fixed with cold acetone for 10 min, rinsed in PBS (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$), overlaid with 0.3 to 0.5 ml of the specific antibody diluted at an appropriate concentration and kept for 30 min at room temperature. After washing, the slides were incubated for 30 min in the presence of the appropriate fluorochrome-conjugated anti-immunoglobulin antibody diluted 1:50, rinsed, and mounted in a glycine-glycerol buffer. In some experiments, the mouse monoclonal antibodies (VmAb and anti-CK55) were used in conjunction with the rabbit polyclonal antibodies (anti-fibronectin, anti-laminin, and anti-collagen) in a double-immunolabeling reaction. In these cases, the frozen sections were first stained with the mouse antibodies using fluorescein-labeled rabbit anti-mouse antibodies as second reagent. The procedure was then repeated with the rabbit antibodies using this time a rhodamine-labeled goat anti-rabbit antibody. Fluorescein isothiocyanate-conjugated rabbit anti-mouse, goat anti-guinea pig, and goat anti-rabbit antibodies were purchased from Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, and from Rego Industries, Québec, Canada. Rhodamine-conjugated goat anti-rabbit antibodies absorbed against mouse IgG were prepared by Dr. Jean-Paul Valet (Laval University Hospital Center, Québec, Canada). Control experiments revealed no cross-reaction between antibodies.

Albumin Production: The rate of albumin production was calculated from measurements made on aliquots of the spheroid medium taken in the same set of dishes over a period of 6 h. The albumin concentration was measured using a double antibody radioimmunoassay as described before (33). Pure rat albumin radiolabeled with ^{125}I by the chloramine T method was a gift of Dr. Luc Belanger.

Tyrosine Aminotransferase (TAT) Activity: TAT activity was determined as described before (32). In the experiments where TAT activity was measured, the cultures were initiated and maintained in Williams' medium E supplemented with insulin (100 ng/ml), epidermal growth factor (100 ng/ml), 3-3′-5-triiodo-L-Thyronine (10 μg/ml), and bovine serum albumin (1%). TAT induction is expressed as the ratio of the activity 9 h after addition of dexamethasone (1 μM) and glucagon (1 μM) to the activity measured at the time of the addition. This time corresponds to maximal stimulation of TAT activity (data not shown).

RESULTS

Spheroid Formation and Development

Freshly isolated hepatocytes, plated in dishes that had been precoated with poly-HEMA, aggregated and, within 1–2 d, formed spheroids of compact morphology with an initial mean diameter of ~50 μm. The photomicrographs of Fig. 1 illustrate the kinetics of spheroid formation. During the first hours of culture, the average multiplicity of the aggregates increased linearly with time reaching an approximate value of 3 cells/aggregate after 5 h (Fig. 2). Up to 12 h, the cells were loosely attached to each other and could be easily separated by gentle pipetting. Starting at this time, however, the weak bonds holding the cells together in aggregates were replaced by tighter bonds in such a way that by 24 h few cells could be released by pipetting (Fig. 2). The aggregates then appeared as more compact structures in which individual cells could hardly be distinguished under phase-contrast microscopy (Fig. 1 *b*). The compaction process terminated by 48 h and little change in general morphology was detected later on (Fig. 1, *c* and *d*), except for a slow and limited increase in diameter. Fig. 3 *a* is a plot of the spheroid diameter versus time for the first month in culture. The liver cell spheroids enlarged from 50 μm at d 1 up to 150 μm after 2 wk. Later on, little increase in size was observed and the diameter finally plateaued between 150–175 μm.

No attempt was made to directly assess whether cell growth was responsible for the increase in size. However, measurements of the total number of spheroids per dish between the second day and the fifth week revealed a gradual decline in number from 2,000 down to 200–300 (Fig. 3 *b*). This suggested that a continuous re-aggregation of small spheroids into larger ones was causing the average diameter increase with time. On the other hand, in the light of the observations to be described

[1] *Abbreviations used in this paper:* CK52 and CK55, 52,000- and 55,000-dalton cytokeratins; poly-HEMA, poly (2-hydroxyethyl methacrylate); TAT, tyrosine aminotransferase.

[2] Marceau, N., L. Germain, R. Goyette, and M. Noel, manuscript submitted for publication.

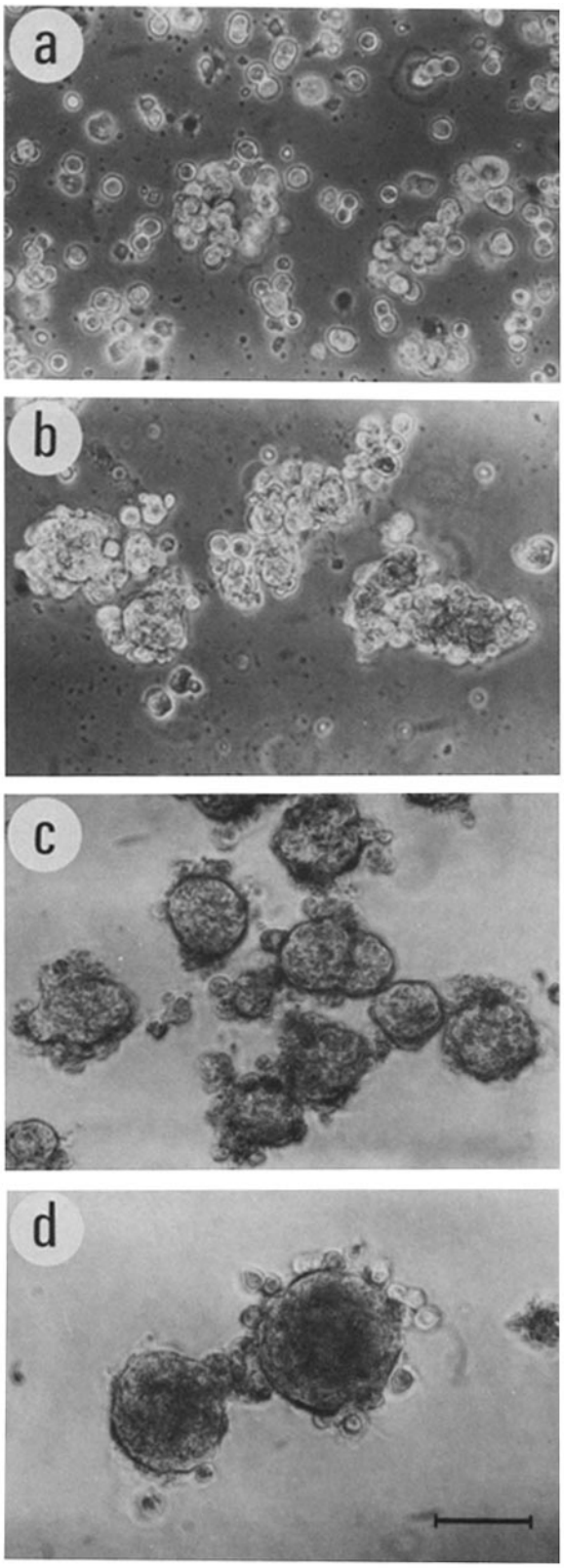

FIGURE 1 Phase-contrast photomicrographs of the liver cell cultures at various times during spheroid formation. (*a–d*) 1 h, and 1, 2, and 5 d, respectively. Bar, 75 μm.

below it is quite clear that growth of some mesenchymal cells also occurred, considering that at the time of isolation the hepatocyte population represented >90% of all cells and that this proportion apparently declined with days in culture.

Cell Sorting Out and Biomatrix Deposition

Histological analyses of spheroids maintained in culture for 10 d to 3 wk revealed the presence of characteristic multicellular structures that reflected a conservation by the dissociated cells of some histogenic potential. At the cellular level, hepatocytes appeared in the aggregates with a fairly normal morphology, showing distinct cell borders, containing one nucleus with one or two dark nucleoli (Fig. 4) and presenting an elaborate cytokeratin network (Fig. 5). Although in some instances these cells were observed directly on the spheroid surface, they were generally found deeper in the aggregate, directly below an external cell lining, where they were arranged in small islands often delimiting a central lumen. As illustrated in Fig. 5, the whole structure so formed was delimited by a thick but discrete zone of deposited extracellular matrix materials containing laminin, fibronectin, and collagen.

A cell type morphologically different from the hepatocytes and forming structures similar to bile ductules in situ was observed in approximately one third of the spheroids (Fig. 6, *a* and *c*). These cells were found to be vimentin negative (data not shown), CK52 positive (Fig. 6*a*), and CK55 positive (Fig. 5), a feature which characterizes the ductular cells in vivo[2] (Fig. 6*b* and references 30, 31). It is therefore suggested that the ductules reformed within the spheroids from ductular cells or ductular cell precursors that were present in the original freshly dissociated liver cell preparation. In support of this notion, chains of ductular structures were seen in some spheroids (Fig. 6*c*) that clearly resembled what is observed in bile ductular cell hyperplasia induced by bile duct ligation in vivo (Fig. 6*d*). Interestingly, the bileduct-like structures in the liver

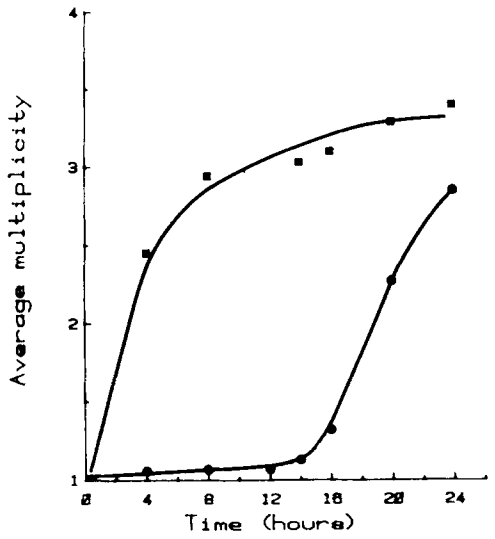

FIGURE 2 Average number of cells per aggregate as a function of time during the first day in culture. The average multiplicity was determined using an electronic particle counter as the ratio of the initial number of cells at time 0 (1×10^6 cells/dish) divided by the number of particles at the time indicated. Particle number was estimated in two different ways to distinguish between loosely aggregated cells and firmly attached cells. The culture dishes containing the cells were poured gently into counting vials and a first estimation of the total number of particles was obtained (loose plus compact cell aggregates: ■). The content of the vials was then pipetted 10 times up and down in a 10-ml pipette and a second counting was made (compact aggregates only: ●).

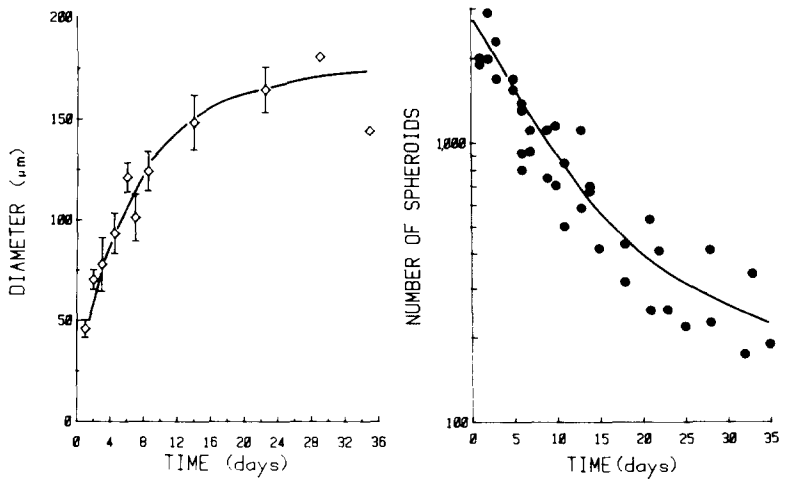

FIGURE 3 (*A*) Spheroid diameter as a function of time in culture. Each data point represents a mean calculated in up to seven independent experiments with at least three dishes per experiment per time point. For each dish the diameter of 30 to 50 spheroids was measured. (*B*) Total number of spheroids per dish as a function of time in culture. Results are from six independent experiments. Each data point represents the mean of 2 to 3 dishes.

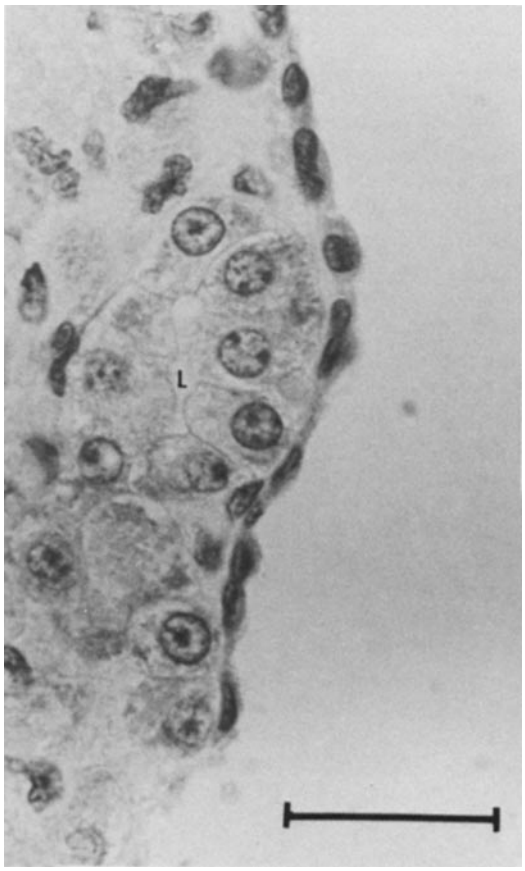

FIGURE 4 Photomicrograph of the edge of a hematoxylin-eosin-stained section of a 200-μm liver cell spheroid at day 11, showing an island of hepatocytes and the presence of elongated cells with dark nuclei forming a lining on a segment of the spheroid surface. A central lumen (*L*) was often observed in hepatocyte islands (see also Fig. 5). Bar, 35 μm.

cell aggregates were in all cases examined delimited by a thick deposit containing laminin, fibronectin, and collagen (Fig. 5). As shown in Fig. 7, this is similar to the in vivo situation in which the three extracellular matrix components are seen in the bile duct wall and in the connective tissue of the portal area.

As mentioned above, the liver cell spheroids were found in most experiments to be covered by an often uninterrupted single cell layer (Fig 4). This cell lining was also visible under low magnification phase-contrast microscopy where it appeared as a thin dark line at the periphery of the spheroids (Fig 1, *c* and *d*). There is a possibility that these cells might correspond to the mesothelial cells lining the external surface of the liver in vivo (Glisson membrane). In addition to the similarity in positioning, the cells seemed to express the same cytoskeleton markers. Indeed, as shown in Fig. 8, the three antibodies monospecific for vimentin, CK52, and CK55 respectively, reacted against the external cell lining of the spheroids; and in parallel experiments, we have observed that this particular combination of markers characterizes the mesothelial lining covering the liver surface in vivo (data not shown) and cultured mesothelial cells.[2] In some cases, however, spheroid linings composed of cells not reacting with one of either markers were also detected (data not shown).

Conservation of morphogenic capacities by dissociated cells has been described before, in most cases for embryonic cells but also in some instances for postnatal cells. For example, postnatal ovarian cells (22), dissociated rat testes cells (23), and thyroids cells (24, 34) were found to reassociate into structures typical of their tissue of origin when cultured under proper conditions. In the case of postnatal liver cells, however, formation of typical tissue structures has been reported after reimplantation of dissociated liver cells in vivo (35), but to our knowledge, has never been described before in an in vitro system.

Hepatocyte Survival

While most of the histological observations reported above were performed with spheroid cultures aged between 1 and 3 wk, observations made at later times revealed the same apparent integrity at the structural level for at least up to 6 wk. A direct assessment of cell survival in spheroids was performed by looking at the ability of the spheroid cells to migrate out of the three-dimensional structures and to spread on collagen substratum after various periods of time in spheroids. The results shown in Fig. 9 indicate that spheroids maintained in culture up to 57 d still contained viable hepatocytes and some nonhepatocytic cells capable of tightly attaching to the substratum.

Maintenance of Hepatocyte Functional Activities

Under conventional monolayer culture conditions, hepatocytes lose rapidly their ability to perform differentiated

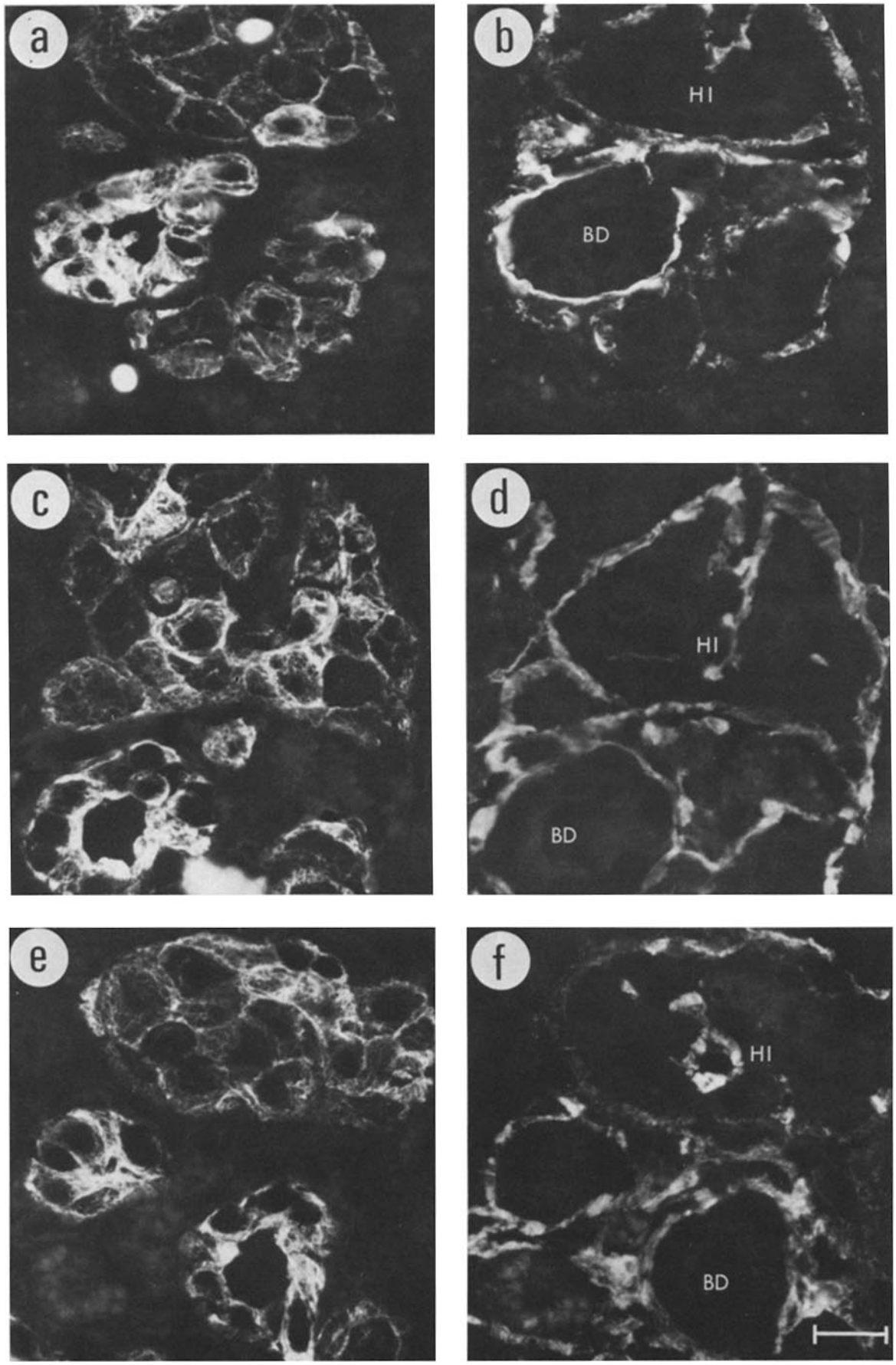

FIGURE 5 Immunofluorescence labeling of CK55 (*a*, *c*, and e), fibronectin (*b*), collagen (*d*), and laminin (*f*) by double-antibody reactions in three sections (a–*b*, *c*–*d*, and e–*f*) of a liver cell spheroid after 8 d in culture. A thick deposit of extracellular matrix materials is observed around hepatocyte islands (*HI*) and bileduct-like structures (*BD*). Bar, *20 μm*.

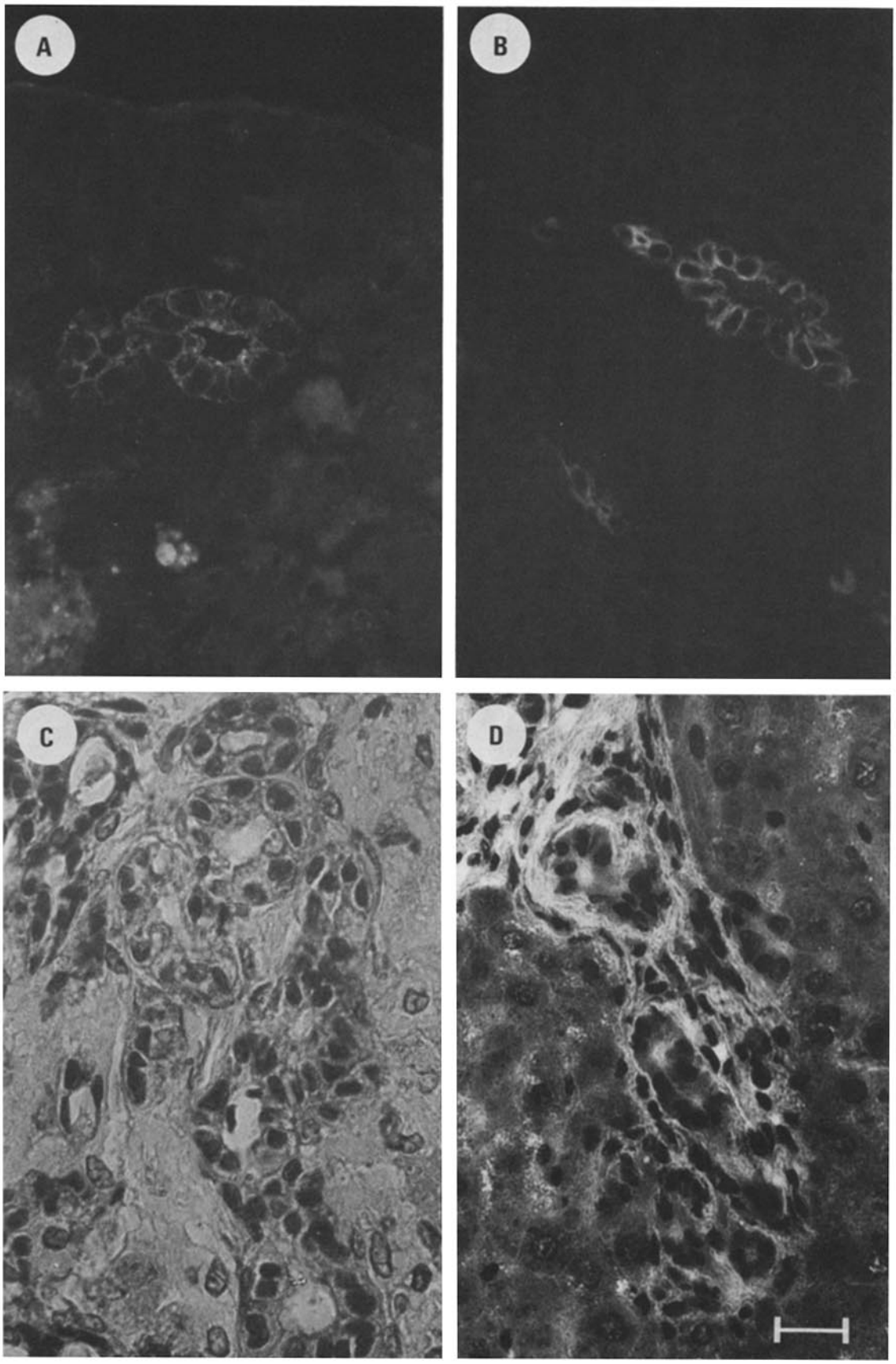

FIGURE 6 Formation of bile ductular structures in the spheroidal aggregate culture. (*A* and *B*) Immunofluorescence labeling of CK52 in a histological section of a liver cell spheroid maintained in culture for 8 d (*A*) and of the liver from a 7-d-old rat (*B*). Note in both cases the specific labeling of the ductular structures with little staining of the surrounding hepatocytes. (*C* and *D*) Hematoxylin-eosin-stained sections of a liver cell spheroid maintained in culture for 23 d (C) and of the liver of an adult rat 10 d after ligation of the common bile duct (see reference 50). The resemblance between the chain of ductule-like structures observed in vitro and those formed in vivo by the hyperplastic bile ductular cells is striking. Bar, 20 μm.

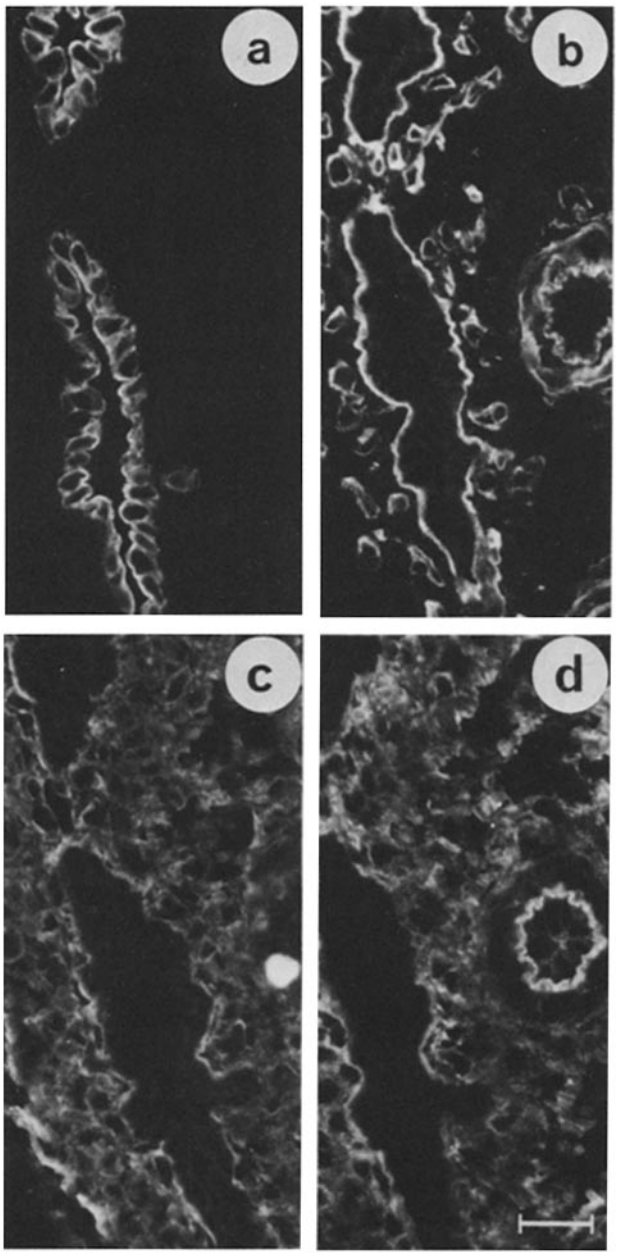

FIGURE 7 Immunofluorescence labeling of CK52 (*a*), laminin (*b*), collagen (c), and fibronectin (*d*) in successive sections of the portal area of the liver from a 13-d-old rat. The three extracellular matrix components are present in the bile duct wall. Collagen and fibronectin are abundant in the region of the field rich in connective tissue. Bar, 20 μm.

functions (4, 5, 14). In an attempt to evaluate the appropriateness of the spheroid system in allowing maintenance of hepatocyte functions, the relative rate of albumin production and the inductability of TAT by glucagon and dexamethasone were measured as a function of time in culture. As shown in Fig. 10, these two hepatic activities were maintained in spheroid cultures at levels corresponding to that measured at 3–5 d (20–50% of day 0) for up to 5–6 wk (no measurement was performed at later times). Parallel determinations performed on hepatocyte preparations maintained in identical medium composition but on collagen substratum (monolayer) revealed a complete loss of TAT activity by 1 wk and of albumin production by 2 wk (data not shown).

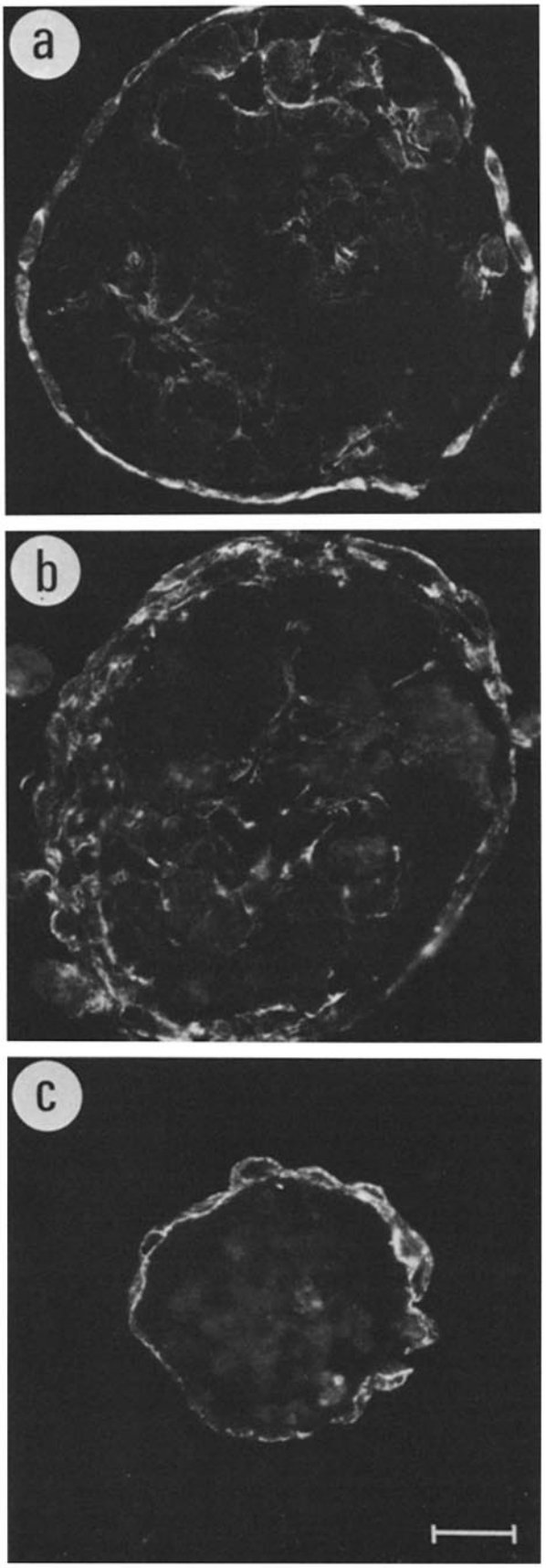

FIGURE 8 External cell lining of spheroids illustrated by immunofluorescence labeling of CK55 (*a*), vimentin (*b*), and CK52 (*c*) in three different histological sections. Note in *b* that few fibroblastic cells (vimentin-positive cells) appear to be present in the aggregates. Bar, 20 μm.

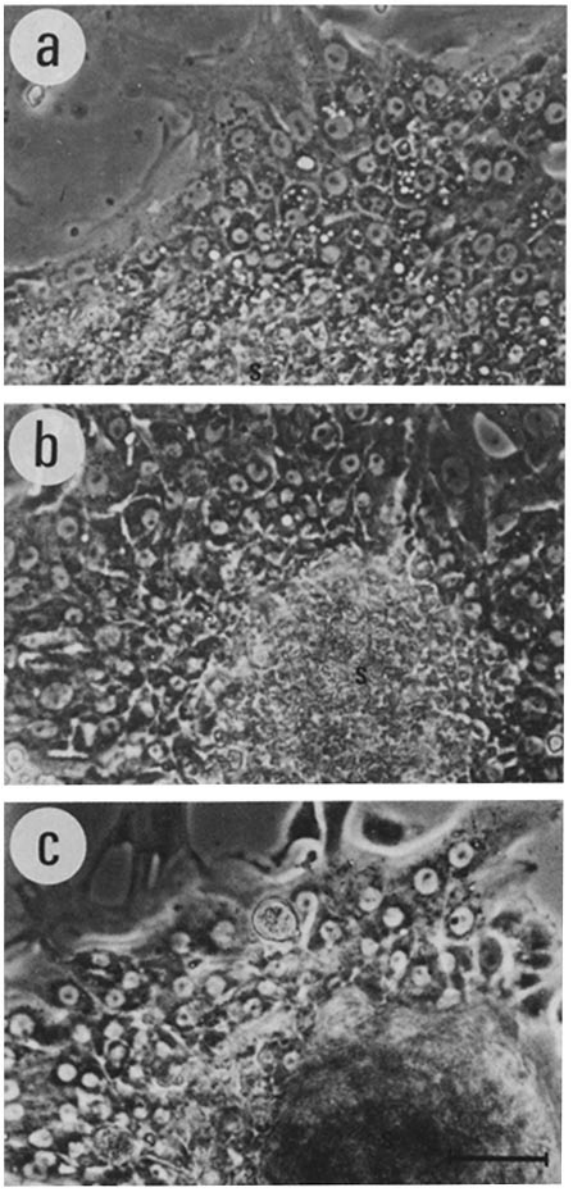

FIGURE 9 Attachment of the spheroid-cultured liver cells to a collagen substratum. The spheroids were maintained in suspension culture for 6 (*a*), 15 (*b*), or 57 d (*c*) and then transferred to collagen-coated dishes. Phase-contrast photomicrographs were taken three d later. *S*, regions of unspread spheroid cells. Bar, 50 μm.

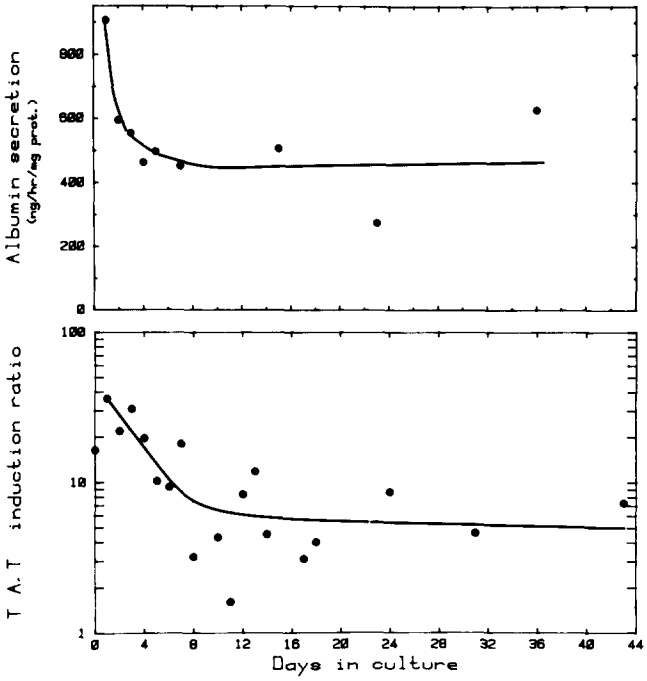

FIGURE 10 Production of albumin (ng/hr per mg of protein) and TAT activity induction by dexamethasone and glucagon by liver cells maintained in spheroid culture for the time indicated.

DISCUSSION

Under the standard monolayer culture conditions, maintenance of survival and of differentiated functions by hepatocytes has proven to be a difficult task. These cells, similarly to many other freshly isolated differentiated cells, hardly recover dispersion from their in vivo environment and require in vitro an appropriate nutritional medium supplemented with a precise combination of humoral and insoluble factors that remains to be defined. It is shown here that when prevented to attach to a solid substratum, liver cells isolated from postnatal rats re-aggregate and re-form structures resembling in some aspects those found in vivo. Under these culture conditions, the hepatocytes can maintain viability and functional integrity for months even in the absence of added serum, or hormone or growth/differentiation factors. It is therefore suggested that liver cells possess and retain upon isolation part of the information required for the formation of a complex tissue architecture and that these special cytoarchitecture features re-formed by the liver cells in the aggregate culture provide to the cells a very appropriate in vivo–like microenvironment which self-generates factors of major importance in the ability of the cell to maintain viability and differentiated functions.

The biochemical basis for cell aggregation, sorting out, and reorganization in three-dimensional structures has been extensively studied with embryonic cells (9, 19, 20, 36–40). With postnatal liver cells, the aggregation process seems to be composed of two distinct phases (Fig. 2). The initial phase extends for up to ~5 h and corresponds to the initial agglutination of the single cells into loosely attached aggregates. This phase, previously termed "phase of recognition" (19), appears to result from direct chemical or physicochemical interactions among specific cell surface determinants. As demonstrated with hepatocytes (Ouellet C., and J. Landry, unpublished results) and also with other cell types (41), this phase is indeed temperature independent and is not affected by inhibitors of protein and RNA synthesis or of energy production. It does require, however, the expression on the cell surface of specific adhesion sites which, in the postnatal liver, appears to be under developmental and hormonal regulation (Ouellet, C., and J. Landry, manuscript in preparation). The second phase in the formation of the liver spheroids begins at ~12 h and is characterized at the macroscopic level by the transformation of the loosely attached aggregates into compact structures. This compaction phase probably results from the formation of specialized junctions between the cells as a correlation has been found in aggregates formed from embryonic cells between the kinetics of increase in the strength of adhesiveness between aggregated cells and the kinetics of formation of these junctions (38). Moreover, with the liver cells (Ouellet, C., and J. Landry, unpublished results) and in other cell systems (41), it has been shown that this phase, contrary to the recognition

phase, is dependent on the presence of calcium, requires protein and RNA synthesis, and also the production of energy.

Little is known concerning the mechanisms of the sorting out of the different cell populations and their organization in typical morphological patterns within the spheroids. The processes most likely result from the interactions of cell-type specific recognition molecules and the differential intercellular adhesive properties of the different cell types (37, 39). The results presented in Fig. 5 may suggest that similarly to its proposed role in the process of embryonic cell migration in vivo (42, 43), biomatrix materials secreted by the cells may also be involved in the morphogenesis of the aggregates. This material is in the liver cell spheroids deposited at specific location between cells, defining precise structural units of apparently homologous cell type and therefore, perhaps, helps in the stabilization of the segregation process. This is likely considering the evidence which suggests the presence of specific receptors for collagen, laminin, and fibronectin on the surface of a variety of cells, including hepatocytes (44–46).

This accumulation and specific deposition of extracellular matrix materials are also likely to be responsible in part for the benificial effect of the aggregate culture on the maintenance of the hepatocytes. In monolayer cultures, the choice of a proper substratum is an important factor for the long-term maintenance of hepatocytes. Reid (4) and Rojkind et al. (47) cultured hepatocytes in monolayer in the presence of an extract from the liver extracellular matrix and detected albumin intracellularly for up to 150 d. In parallel experiments, they further showed that components isolated from other organs were much less efficient in supporting hepatocyte survival. In aggregate cultures, it may be postulated that a similarly active liver-specific material can be regenerated by the liver cells, a capacity that would be for some reasons not expressed in monolayer. Another possibility is that, in addition to an appropriate biochemical composition of the insoluble materials, a precise geometrical deposition of this material relative to the cells and around cellular structures (in the present case, bile ducts and hepatocyte islands as shown in Fig. 5) may be important for the maintenance of the cells. In support to this, Grover et al. (48, 49) recently demonstrated that the expression of specific visceral endoderm functions by F9 embryocarcinoma cells cultured in aggregates was dependent on the formation of a proper basement membrane. They showed that not only laminin and fibronectin produced by the cells were essential for their differentiation, but moreover, that the insoluble proteins had to be deposited in a very specific pattern relative to the epithelial cells.

An additional factor of likely importance in the prolonged maintenance of hepatocytes in aggregate culture may be the higher level of homologous and heterologous cell–cell contacts rendered possible in a three-dimensional structure. It is well acknowledged that a variety of positive as well as negative signals regulating growth and differentiation can be provided by intercellular contacts (9). In agreement with observations in other cell systems, there seemed to be little fibroblastic growth in the hepatocyte aggregates (Fig. 8 *b*), whereas the same cell preparations set up in monolayer culture are rapidly overgrown by fibroblastic cells (data not shown). It is likely, however, that other cell types such as the "ductular" cells multiply a few times in the aggregates. Indeed, although we did not measure the proportion of the various cell types as a function of time in culture, the proportion of hepatocytes which initially was ~90% appears to have decreased substantially in the histological sections studied. It is possible that via a proper growth control of the different cell populations, an appropriate balance is reached so that a quasi-optimal heterologous cellular microenvironment build up around the parenchymal cells. It has indeed been demonstrated that hepatocytes can be maintained in monolayer much longer when other specific cell types are added to the culture. Clement et al. (13) and Guguen-Guillouzo et al. (14) found that human or rat hepatocytes, co-cultured in monolayer with low-differentiated epithelial cells isolated from the liver but not from other organs, survived for more than 2 mo and maintained during this period a high level of expression of albumin secretion. Similar results were obtained when a nonparenchymal liver cell line apparently of endothelial origin was used in the co-cultures (Morin, O., personal communication). In these experiments, the prolonged survival was attributed in part to appropriate heterologous cell interactions and also to the production by the co-cultured cells of proper extracellular matrix materials.

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