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Ultrastructural and Functional Differentiation of Hepatocytes Under Long-Term Culture Conditions

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ABSTRACT ***Background:*** **Studies on hepatocytes grown in different culture systems have shown that these cells rapidly dedifferentiate on a single support with liquid medium on top (single gel technique). However, in systems sandwiching them between two layers of extracellular matrix (double gel technique), the cells are able to regain and maintain typical light microscopical appearance and function. Their ultrastructural morphology is as yet unknown.**

Methods: **Isolated, adult rat hepatocytes were grown in both systems, and their fine structure (thin section electron microscopy) and the functional ability of albumin production (immunoassay) were studied and compared in both culture systems after 2,7, and 14 days.**

Results: **The hepatocytes in conventional *single gel culture* did not completely regain their normal morphology and rapidly underwent progressive dedifferentiation. This was characterized by loss of cell polarization in terms of obliteration of the bile canaliculi-like intercellular expansions, loss of cell membrane differentiations, and reduction of organelles. Cytoskeletal components gradually increased, building up large filamentous zones underneath the plasma membrane. In *double gel culture,* the hepatocytes reachieved and maintained intact morphology and polarity over at least 14 days. The bile canaliculi were formed, preserved, or even enlarged and were associated with dense peribiliary bodies and Golgi fields. The plasma membrane facing both collagen layers bore numerous cytoplasmic microprojections like the sinusoidal surfaces of the hepatocytes in situ. Cell organelles, glycogen particles, and lipid droplets were always present.**

Conclusions: **The hepatocyte is a cell type in which ultrastructural and functional differentiation are strongly interdependent. For these cells, the morphological microenvironment (i.e. the bipolar *position* of the extracellular matrix) may be as important or even more decisive for maintenance of normal cell differentiation than modifications of the *composition* of the matrix itself or addition of other cell types, as focused in other studies.**

Key words: Hepatocyte differentiation, Hepatocytes in single gel cultures, Ultrastructure of differentiation, Hepatocytes in double gel cultures, Ultrastructure of hepatocytes in culture, Hepatocytes and extracellular matrix

Hepatocytes are still grown, as any other cell type, in conventional culture systems, i.e., on a "single" support (usually a gel or a membrane) with liquid medium on top. However, this procedure ignores basic principles of their normal microenvironment in situ such as their enclosure in liver plates flanked on both surfaces by the extracellular matrix inside the space of Disse. Good morphological and functional data about hepatocytes can be collected by using this established culture sys-

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This paper is dedicated to professor Dr. med. Enrico Reale on the occasion of his retirement.

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tem, but only for a period of some days (Bissel et al., 1973; Chapman et al., 1973; Wanson et al., 1977).

In the present work, a double gel culture system (Dunn et al., 1989; Bader et al., 1992) was chosen, allowing the bipolar attachment of hepatocytes to extracellular matrix in the form of two layers of rat tail collagen, covering the cells from both sides and hence topografically mimicking the in situ microenvironment. Other systems (Guguen-Guillouzo et al., 1983) are approaching this goal by the more complicated use of cocultures with other cell types.

Light microscopic observations on the double system have revealed that it is superior in achieving and maintaining a normal cell shape (Dunn et al., 1989) and a typical expression of cell membrane markers (Musat et al., 1993). These normal features permit long-term adequate functioning of the hepatocytes as demonstrated, e.g., by intact expression of cytochromes (Sidhu et al., 1993), stable albumin secretion (Dunn et al., 1989, 1992), and the ability of acute phase reaction *(Bader et al., 1992)*.

Previous studies dealing with sandwich techniques for primary hepatocyte culture used different compositions of extracellular matrix as a culture support, such as rat tail tendon collagen (Dunn et al., 1989, 1992; Bader et al., 1992; Ezzell et al., 1993), conventional type 1 collagen (Sidhu et al., 1993), or purified monomeric type 1 collagen (Musat et al., 1993). As a culture overlay serves either the same collagenous matrix (Dunn et al., 1989, 1992; Bader et al., 1992; Ezzell et al., 1993) or an enriched basement membrane-like matrix derived from the Engelbreth-Holm-Swarm tumor (matrigel, Musat et al., 1993). However, these investigations focused on functional parameters (Bader et al., 1992; Dunn et al., 1992) or were mainly restricted to light microscopical aspects of the cells (Dunn et al., 1989; Musat et al., 1993; Ezzell et al., 1993; Sidhu et al., 1993). A detailed analysis of the ultrastructural morphology underlying the observed structural and functional hepatocellular changes in the sandwich system with proceeding time in culture was as yet not available.

Therefore, in the present study, hepatocytes in conventional "single gel" and in "double gel" sandwich, both grown on or between membranes or rat tail tendon collagen, were investigated by transmission electron microscopy after different times in culture. Changes of the fine structure of their cytoplasmic organelles, their junctional complex, their cytoskeletal components, and their plasma membrane differentiations toward the "sinusoidal" and the "biliary" surfaces were compared. To demonstrate one of their functional abilities, the albumin secretion of these cells was investigated by enzyme-linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

Cell Culture

Adult rat hepatocytes were isolated as already described (Seglen, 1976) and 2×10^6 cells grown in 60 mm Petri dishes either on one layer (single gel culture) or between two layers (double gel culture) of rat tail tendon collagen. The collagen layers were prepared according to Elsdale and Bard (1972). Hepatocytes were enclosed within two layers of collagen in a modification of the method described by Dunn and coworkers (1989). Thirty minutes after surface coating of the Petri dishes with collagen, hepatocytes were seeded. Four hours later, on top of the adhering cells a second layer of liquid and ice-cold collagen was pipetted. After gelation, culture medium was added containing fetal bovine serum (10%; Biochrom, Berlin, Germany), prednisone (9.6 μg/ml), and glucagon (0.014 μg/ml; both from Novo, Mainz, Germany), insulin (0,16 U/ml; Hoechst, Frankfurt, Germany), glutamate and penicillin/streptomycin (Biochrom). The culture medium was changed daily.

Ultrastructure Analysis

Hepatocytes were fixed immediately after isolation or after periods of 2,7, and 14 days of growth in the two culture systems. Fixation was performed by immersion in glutaraldehyde (2.5% in 0.1 M cacodylate/HCl buffer, pH 7.4) on the culture dishes immediately after removal of the culture medium. The specimens were postfixed with 2% OsO_4 in the same buffer for 1 hour and dehydrated in graded alcohols. Samples of ~ 3 × 5 mm area, containing cells and the respective collagen layer(s) were cut out, removed from the culture dishes, and embedded in epoxy resin (Epon®). Sections were cut orthogonally to the cell layer in order to show the cells in cross section. Thin sections were stained with uranyl acetate and lead citrate and observed in a Zeiss EM 10 transmission electron microscope.

Protein Secretion

The total albumin secretion (in μg per day and per million cells) was measured in the two culture systems after 2, 7, and 14 days. This was performed by an enzyme-linked immunosorbent assay (ELISA). Chromatographically purified rat albumin and antibody were purchased from Cappel (Cochranville, PA); 96-well plates (Nunc, Wiesbaden, Germany) were coated with 1 mg/ml of albumin and left overnight at 4°C. After washing each plate four times, 50 μl of cell culture supernatant were added to the wells; 50 μl of antibody conjugate (anti-rat albumin conjugated with horseradish peroxidase) was added. Following incubation for 24 hours at 4°C, substrate (O-phenylenediamine dihydrochloride, OPD, Sigma, Deisenhofen, Germany) and H_2O_2 were added for 6 minutes. The reaction was stopped by adding 100 μl of 8N H_2SO_4. Absorbance was measured at 490 nm using a Dynatech M5000.

RESULTS

Hepatocytes Immediately after Isolation

The cells were spherical and measured ~ 14–25 μm in diameter. They were covered by irregularly distributed, ~ 0.5 μm long microvilli- and microplicae-like cytoplasmic projections, and the organelles showed a characteristic almost circular arrangement around the central roundish nucleus (Fig. 1). A peripheral cytoplasmic layer was solely occupied by tubules of the smooth endoplasmic reticulum with a few intermingled glycogen particles. The flattened cisternae of the rough surfaced endoplasmic reticulum, isolated and arranged in stacks, were usually concentrated around the nucleus. In an intermediate zone between this and the peripheral layer, small Golgi complexes were intermingled among mitochondria of rather uniform size, peroxisomes, dense bodies, and lipid droplets. In the nucleus the euchromatin was abundant and the nucleolus

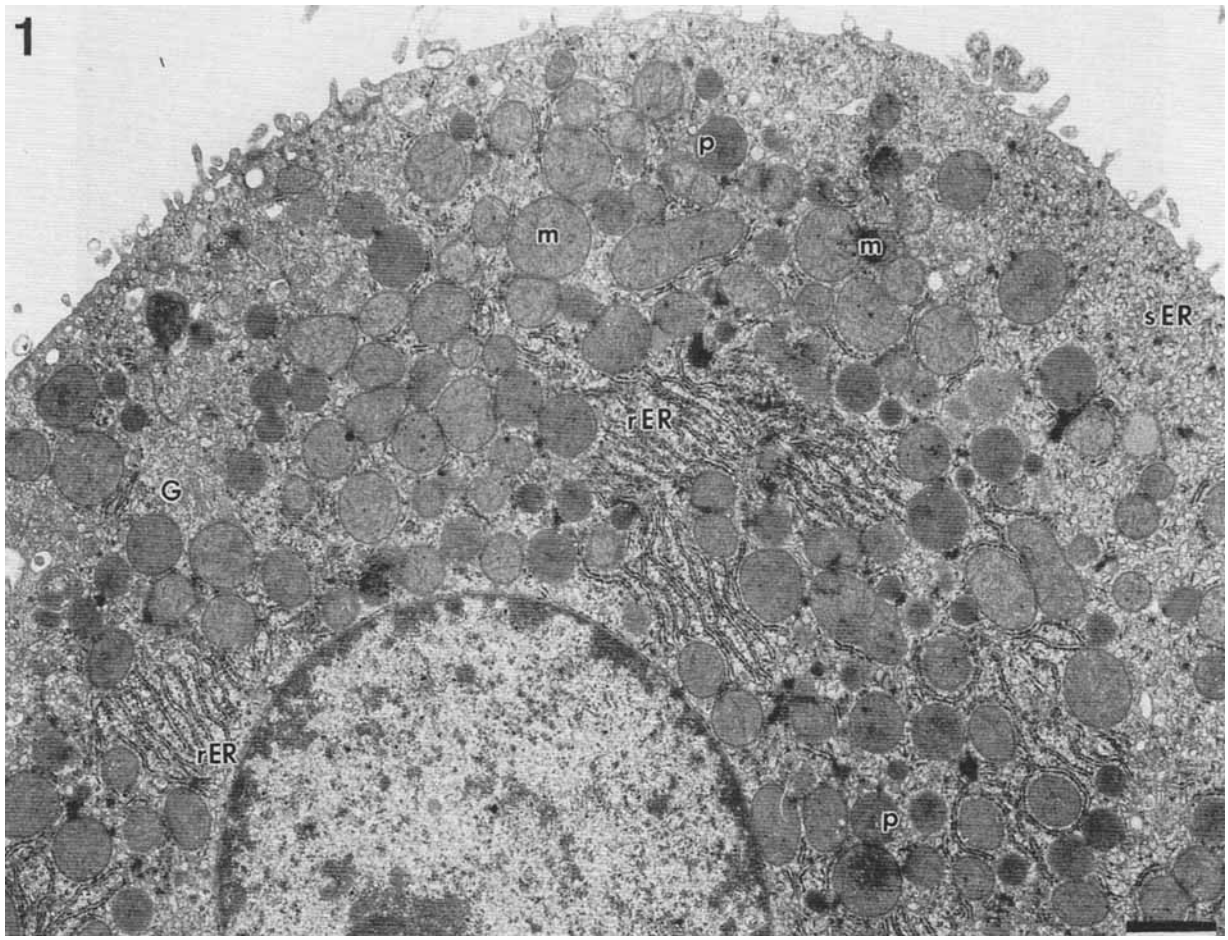

Fig. 1. Part of an hepatocyte immediately after isolation. The spherical cell shows numerous short cytoplasmic microprojections on its surface, a cortical layer of smooth endoplasmic reticulum (sER), and between this and the nucleus, other cell components such as Golgi complex (G), mitochondria (m), peroxisomes (p), rough endoplasmic reticulum (rER), smooth endoplasmic reticulum (sER). ×12,000; bar = 1 μm.

was large. Components of the cytoskeleton were only seldom identified.

Day 2 in Culture

Hepatocytes in single gel culture were already relatively flat showing in the section ~ 1/4–1/3 of the diameter of the isolated spherical cells and overlapping each other to a major extent. Between the hepatocytes there were circumscribed expansions of the extracellular space (Fig. 2A). These occurred in approximately the same position as the bile canaliculi in situ. However, in contrast to the bile canaliculi, their profile was usually elliptical and looked like flattened cisternae rather than tubules. The short diameter of these was about the same size as normal bile canaliculi in situ (0.5–1.5 μm; Weiss, 1988), but the long diameter was much wider. They showed only sparse microvilli-like luminal projections inside and were expressed along most cells but not all. The bile canaliculi-like expansions were closed by junctional complexes like those normally occurring around bile canaliculi in situ, but they could show a different order and extension of their components. For example, desmosomes were scattered over the whole distance of the intercellular space, keeping this narrow all the way from the bile canaliculi-like expansion toward the sinusoidal surface (Fig. 2B,C). Evidence of pentalaminar structures indicating a real sealing of the intercellular space was occasionally found; gap junctions were rarely seen. Finger-like cytoplasmic processes interdigitating adjacent cells were more frequently observed than in situ.

Phagosomes were extraordinarily frequent in these hepatocytes, scattered throughout the cytoplasm (Fig. 2D). Their size varied greatly (from 0.1 to 2 and more μm) as well as their contents and therefore their electron density. They contained fluffy material (like that occasionally found extracellularly among the collagen fibrils), remnants of organelles and myelin (lipid) figures. Smaller and highly electron dense phagosomes were frequently assembled around the intercellular expansions (Fig. 2A) like the peribiliary bodies in situ. Golgi fields were also preferably seen in this area. The numerous mitochondria, excluded from the peribiliary zone, showed a considerable inhomogeneity in size

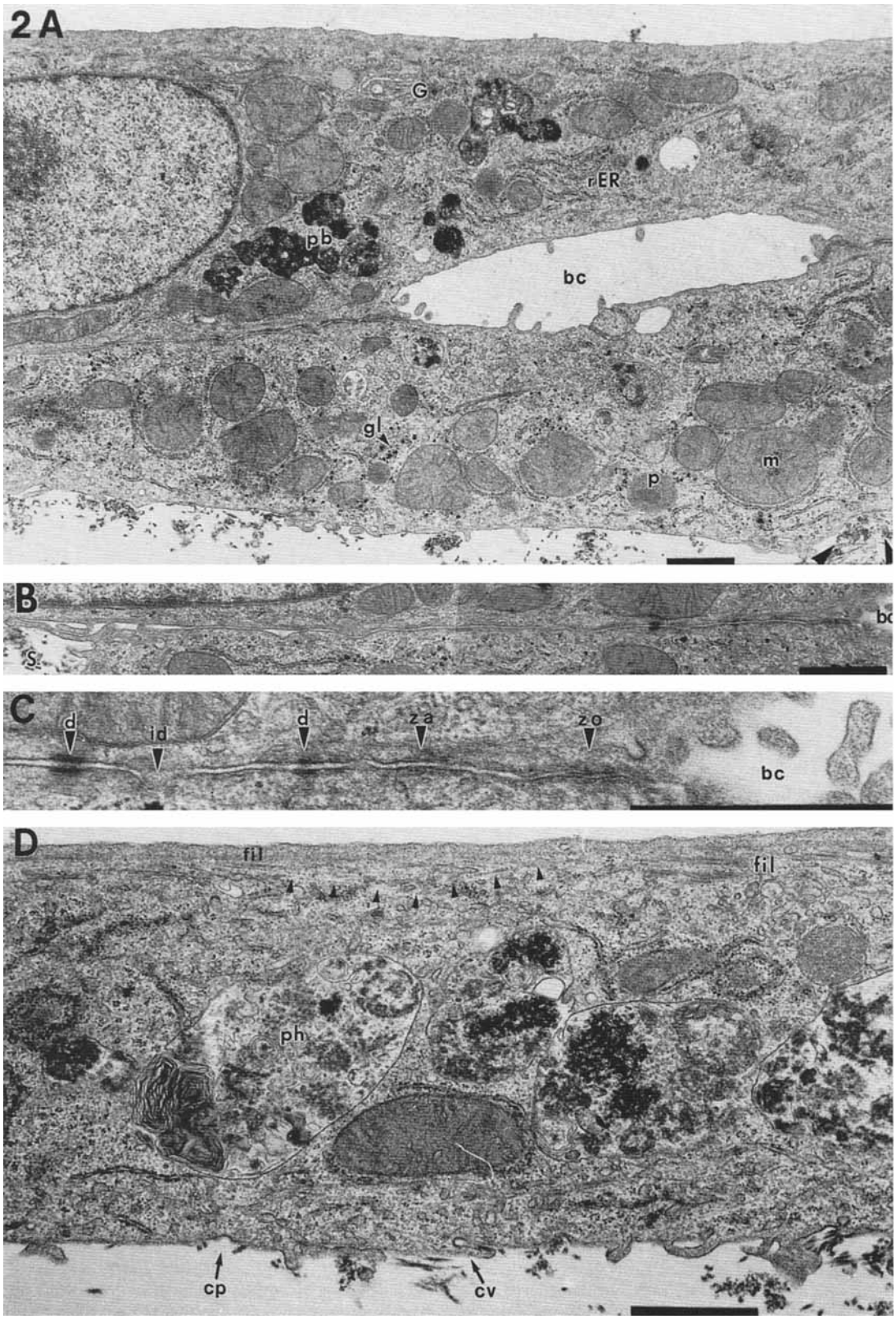

Fig. 2. Hepatocytes in conventional single gel after 2 days in culture. In **A,** two flattened cells with broad overlapping enclose a large bile canaliculus-like intercellular expansion (bc) with sparse microprojections inside. Rough endoplasmic reticulum (rER), mitochondria (m), peroxisomes (p), Golgi complex (G), and numerous peribiliary bodies (pb) of different size, glycogen particles (gl); arrowheads indicate extracellular material. In **B,** the intercellular space is narrow from the bile canaliculus-like expansions (bc) until the free cell surface (S). In **C** (higher magnification of B), two desmosomes (d), a finger-like interdigitation (id), zonula adherens (za), and zonula occludens (zo) toward the bile canaliculus-like expansion (bc). In **D,** filament bundles (fil) and microtubules (arrowheads) prevail underneath the cell surface toward the medium. Huge phagosomes (ph) have different content, partially similar to the extracellular material (arrowheads in A) or to organelle remnants. Coated pits (cp) and coated vesicles (cv) are at the cell face toward the collagen. A ×12,000, B ×16,000, C ×50,000, D ×24,000, bar = 1 μm.

(0.3–3.3 μm) (Fig. 2A). Peroxisomes with a dense core, smooth and rough endoplasmic reticulum occurred in usual morphology. Glycogen particles as well as lipid droplets were rare.

The hepatocytes bore numerous microprojections on the inferior (collagen) side, whereas their other side toward the liquid medium was usually smooth (Fig. 2A,D). Coated pits and vesicles as well as smooth surfaced caveolae were preferably found at the collagen surface (Fig. 2A,D), along the intercellular space and around the bile canaliculi-like expansions (Fig. 2B,C). Underneath the plasma membrane toward the liquid medium, there frequently was a thin dense zone (about a few hundred nm thick) filled with filaments and free of organelles (Fig. 2A,D). Microtubules bordered this filamentous layer in parallel arrangement and were interspersed across the whole cytoplasm (Fig. 2B).

Hepatocytes in double gel culture showed a higher diameter (corresponding to about one-half of the diameter of isolated spherical hepatocytes) and were therefore considerably higher than the cells in conventional single gel culture. The intercellular spaces were mostly upright without major overlapping but with short interdigitating cytoplasmic processes (Fig. 3A). The intercellular expansions were about the same size but less elliptic than in single gel culture and resembled more the bile canaliculi in situ. They also had few microvilli and occasionally showed septum-like cytoplasmic folds ranging into the lumen. Different from the single gel culture, bile canaliculi-like expansions were found at each of the intercellular spaces between adjacent hepatocytes, hence occurring at regular intervals in the cross sections. Tight junctions were more frequently identified in this type of culture system than in the single gel, but also not restricted to the peribiliary region and seen toward the "sinusoidal" side of the intercellular space as well (Fig. 3B).

The cells showed a normal distribution of organelles with plenty of dense bodies and Golgi fields in the peribiliary zone (Fig. 3A). Numerous mitochondria of rather homogeneous size (0.5–1.5 μm), smooth endoplasmic reticulum, peroxisomes with a dense core, glycogen particles, and lipid droplets (Fig. 3A) were found in the remaining cytoplasm. Compared to the single gel culture, there were only few phagosomes.

Numerous microprojections, coated pits, and vesicles were found on both sides of the cells toward the collagen layers as in the sinusoidal surface of hepatocytes in situ and in contrast to those in the conventional single gel culture.

Day 7 in Culture

In single gel culture, the bile canaliculi-like expansions of the intercellular space were less frequently observed and the hepatocytes were usually only connected by zonula adherens, desmosomes, and interdigitations without recognizable zonula occludens. The amount of organelles, and in particular of the mitochondria, was decreased. The mitochondria were frequently large, with a rather electron-dense matrix and sparse, narrow, short cristae (Fig. 4A). Also, the typical polarized distribution of the organelles inside the hepatocytes was less evident than on day 2.

The peripheral filamentous zone underneath the plasma membrane bordering the medium was thicker now and also showed dense spots (Fig. 4A,B). Toward the cytoplasm, this zone was accompanied by distinct bundles of intermediate filaments (Fig. 4B) and microtubuli (Fig. 4B,E); centrioles were also detectable (Fig. 4A). A similar filamentous zone, but inconstant and thin, also could be present toward the collagen (Fig. 4A). Microvilli and microplicae-like cytoplasmic projections were reduced in number and preferably occurred around the cell contact zones. Small sheets of finely granular (basal lamina-like) material were occasionally seen directly underneath the cell surface toward the collagen (Fig. 4A). Higher magnifications (Fig. 4C,D) show these to be opposed to intracellular areas of increased density associated with intracellular microfilaments. Sometimes, intracellular filaments were seen there colinear with extracellular filaments (Fig. 4E).

In double gel culture, the bile canaliculi-like expansions were found in unchanged frequency but often with increased diameter and occasionally subdivided by septum-like cytoplasmic structures (Fig. 5). They were limited by complete junctional complexes. Around the expansions, dense material occurred along the cell membrane inside adjoined cells, probably indicating an enlarged extension of the zonula adherens (Fig. 5). The amount of cell organelles in general was not reduced. The mitochondria could show an inhomogeneity in size (ranging from ~ 0.5–4 μm) but were morphologically intact. Cytoplasmic microprojections remained numerous on both surfaces facing the collagen layers together with numerous coated pits and vesicles.

Day 14 in Culture

In single gel culture, bile canaliculi-like expansions were no longer detectable between the flattened hepatocytes. At the long overlapping contact zones of adjacent cells, only desmosomes remained as junctions, accompanied in the intercellular space by coated pits and vesicles (Fig. 6A).

The organelles were extremely rarefied and usually structurally altered. This was especially true for the mitochondria that had still an increased electron density, sparse cristae, and frequently irregular shape; sometimes it was even difficult to differentiate them from dense phagosomes (Fig. 6A,B,C).

Most of the remaining cytoplasm was filled by these dense phagosomes together with elements of the cytoskeletal system. Major areas, occupying up to one-half of the cell height (Fig. 6B) toward the liquid medium were filled with filaments forming meshworks with dense spots (Fig. 6B,C), sometimes assembled in clearly defined bundles. Microtubules were as well detectable in larger amounts in the vicinity (Fig. 6C,D,E). Smooth cisternae, most likely of the Golgi apparatus, were still found in considerable amounts (Fig. 6C).

Cytoplasmic microprojections on both sides of the cell had almost completely disappeared. Basement membrane material underneath the cells, sometimes opposed to dense intracellular areas, was frequently observed (Fig. 6D,E) as before in the 7-day single gel cultures.

The hepatocytes in the double gel system could show increasing overlapping, although the height of the trabecles they formed remained unchanged. The fre-

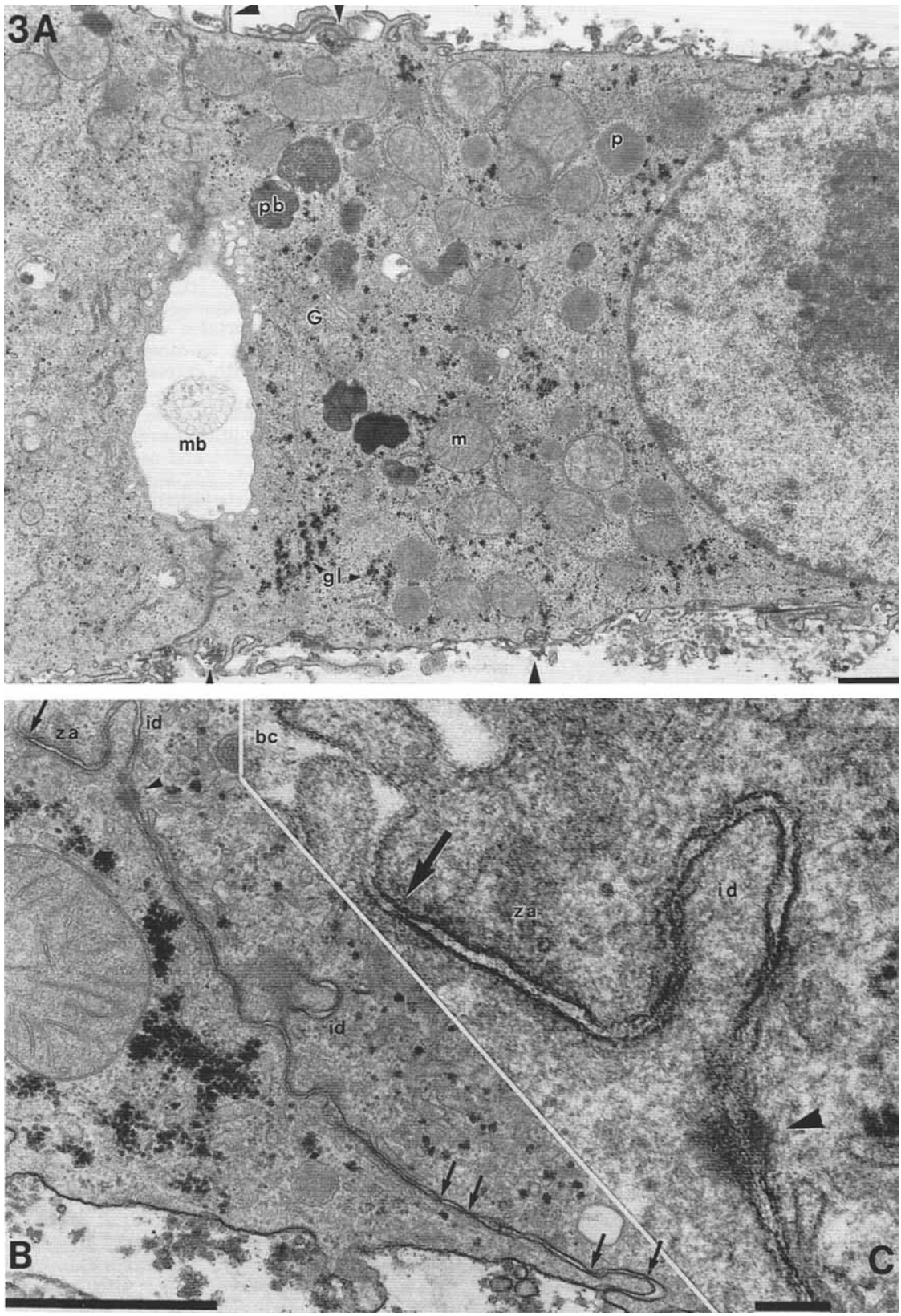

Fig. 3. Hepatocytes after 2 days in the double gel culture. The cells have polyhedral shape and normal distribution of organelles. Peribiliary bodies (pb) and Golgi fields (G) around a bile canaliculus-like expansion containing a multivacuolar body (mb); mitochondria (m), peroxisomes (p), and glycogen particles (gl). On both cell surfaces, numerous microprojections and between them, granular extracellular material (arrowheads). **B**. Narrow convoluted intercellular space with finger-like cytoplasmic interdigitations (id), zonulae occludentes (arrows), zonula adherens (za), and a desmosome (arrowhead). Flattened cisternae of the endoplasmic reticulum flank occasionally the intercellular space. In **C** (higher magnification of B), a junctional complex with pentalaminar fusion of the adjacent cell membranes (zonula occludens, arrow) near bile canaliculus-like expansion (bc); desmosome (arrowhead) and cytoplasmic interdigitation (id). A $\times$12,000, B $\times$40,000, bar = 1 μm, C $\times$140,000, bar = 0,1 μm.

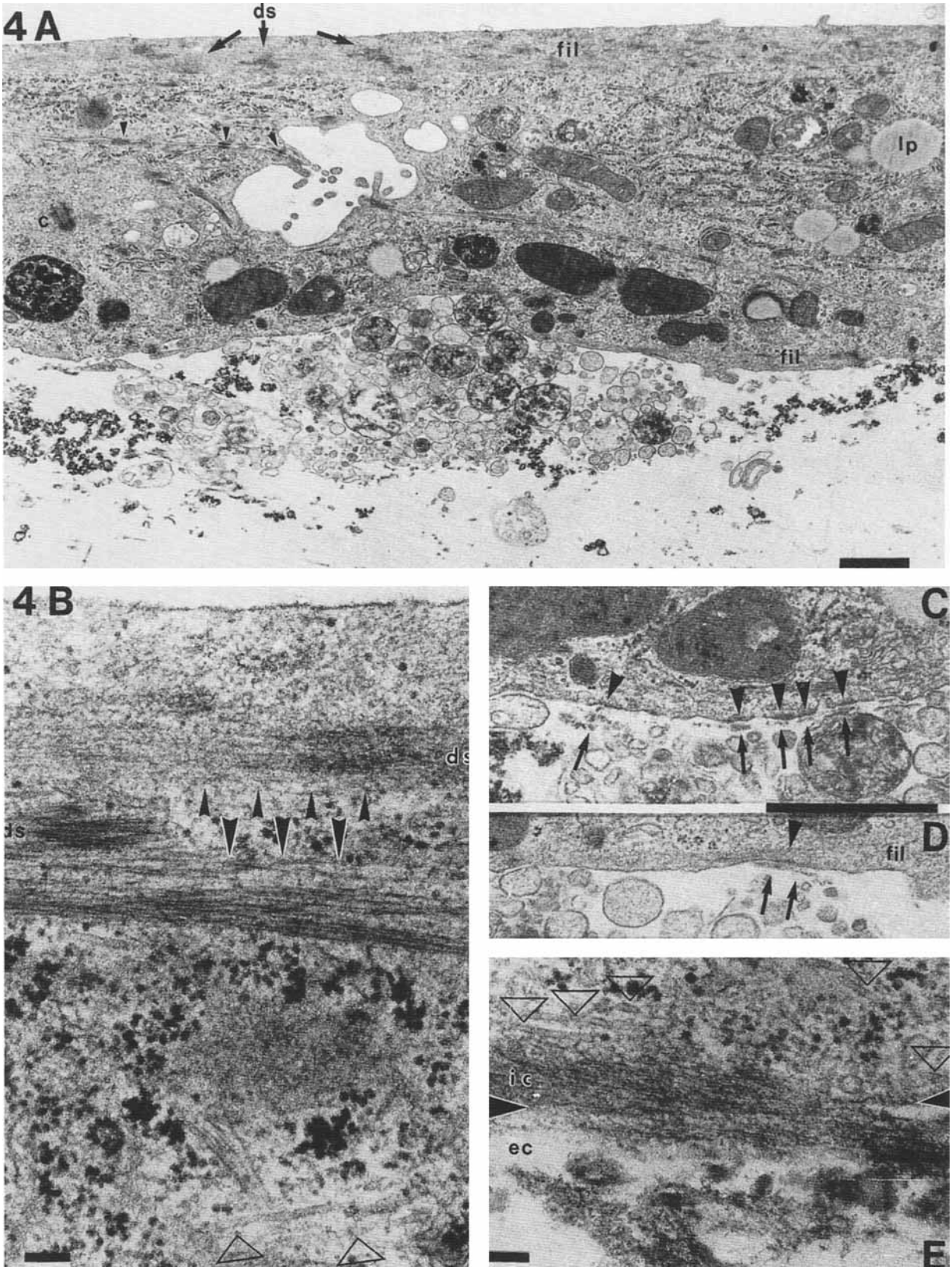

Fig. 4. Hepatocytes after 7 days in single gel culture in **A** with extensive overlapping, connected by numerous desmosomes (arrowheads). Lipid droplets (lp); centriole (c); thick filament layer (fil) toward the liquid medium with interspersed dense spots; smaller filament zone (fil) on the opposite side; only few microprojections. Abundant cell debris in an excavation underneath the cells. In **B,** microfilaments (small arrowheads) with dense spots (ds), bundles of intermediate filaments (large arrowheads), microtubuli (open arrowheads). In **C** and **D** (higher magnifications of A), basement membrane material (arrows) opposed to dense intracellular areas (arrowheads). In **E,** the intracellular microfilaments (ic) are colinear with extracellular filaments (ec); dark arrowheads indicate the level of the cell membrane; open arrowheads at microtubuli. A ×12,000, C and D ×30,000, bar = 1 μm; B and E ×80,000, bar = 0.1 μm.

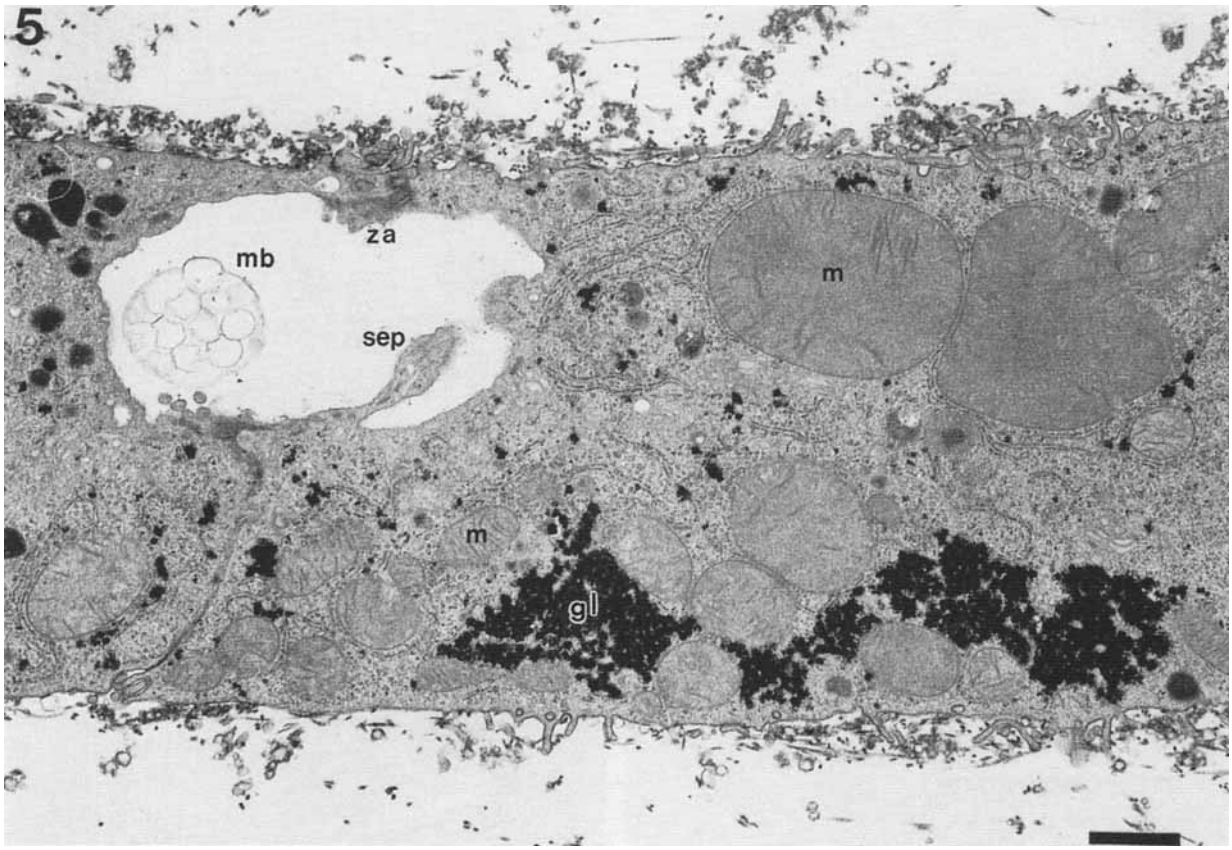

Fig. 5. After 7 days in double gel culture. The expansion of the intercellular space contains a large, septum-like, cytoplasmic protrusion (sep) and a multivesicular body (mb). Filamentous zones (parts of extensive zonulae adherentes, za) at the intercellular space close to the bile canaliculus-like expansion. Mitochondria (m), peribiliary bodies, and glycogen (gl). The microprojections are still frequent on both cell surfaces with numerous coated pits and vesicles in between. ×12,000, bar = 1 μm.

quency of bile canaliculi-like expansions between the hepatocytes remained stable, but they were usually cavernously enlarged, bordered by more than two cells and encircled by distinct filament layers (Fig. 7A,B). In increasing frequency, at cell contacts, preferably near the bile canaliculi-like expansions, there were large zones with a dense filamentous membrane coat representing extensive zonulae adherentes (Fig. 7C). The amount of organelles appeared slightly reduced and the mitochondria could show an inhomogeneity of size, but they appeared distinctly more normal than in the conventional single gel cultures of the same age.

Elements of the cytoskeleton were not predominating as in the conventional single gel culture. Microfilamentous bundles were solely found around large bile canaliculi-like expansions, and occasional distinct bundles of intermediate filaments could be scattered throughout the cytoplasm (Fig. 7B,E). Glycogen particles were still frequent, also in larger accumulations, and lipid droplets as well. Cytoplasmic microprojections and coated pits and vesicles were numerous at both hepatocyte surfaces lined by collagen, as found in hepatocytes in situ in the space of Disse.

Albumin

Cumulated albumin secretion for each respective day in the two different culture systems (Fig. 8) is very similar (with ~ 18 μg/10^6 hepatocytes) in both types of cell culture at 2 days. After 7 days in culture, the hepatocytes grown in conventional single gel culture have a drastic decrease of albumin secretion to 1 μg/day/10^6 cells, whereas those grown in the double gel system produced albumin at 36 μg/day/10^6 cells. Another week later (day 14 in culture), the functional aspect of albumin secretion is almost reduced to zero in the single gel, whereas it shows a steady increase in double gel culture.

DISCUSSION

Starting from a completely altered configuration with total loss of cell polarity caused by the isolation procedure (as shown here and by Berry and Friend, 1969; Wanson et al., 1977; McMillan et al., 1988), the hepatocytes in both culture systems regained some basic aspects of their usual ultrastructure and polarity. They reconstituted bile canaliculi-like expansions, intercellular junctions, and polarized distribution of organelles (i.e., peribiliary bodies, Golgi fields). But already after 2 days, the hepatocytes in the single gel missed, e.g., microprojections on both "sinusoidal" surfaces, upright lateral surfaces, and the regular distribution of bile canaliculi-like structures, as expressed in situ and regained in double gel culture. From then on,

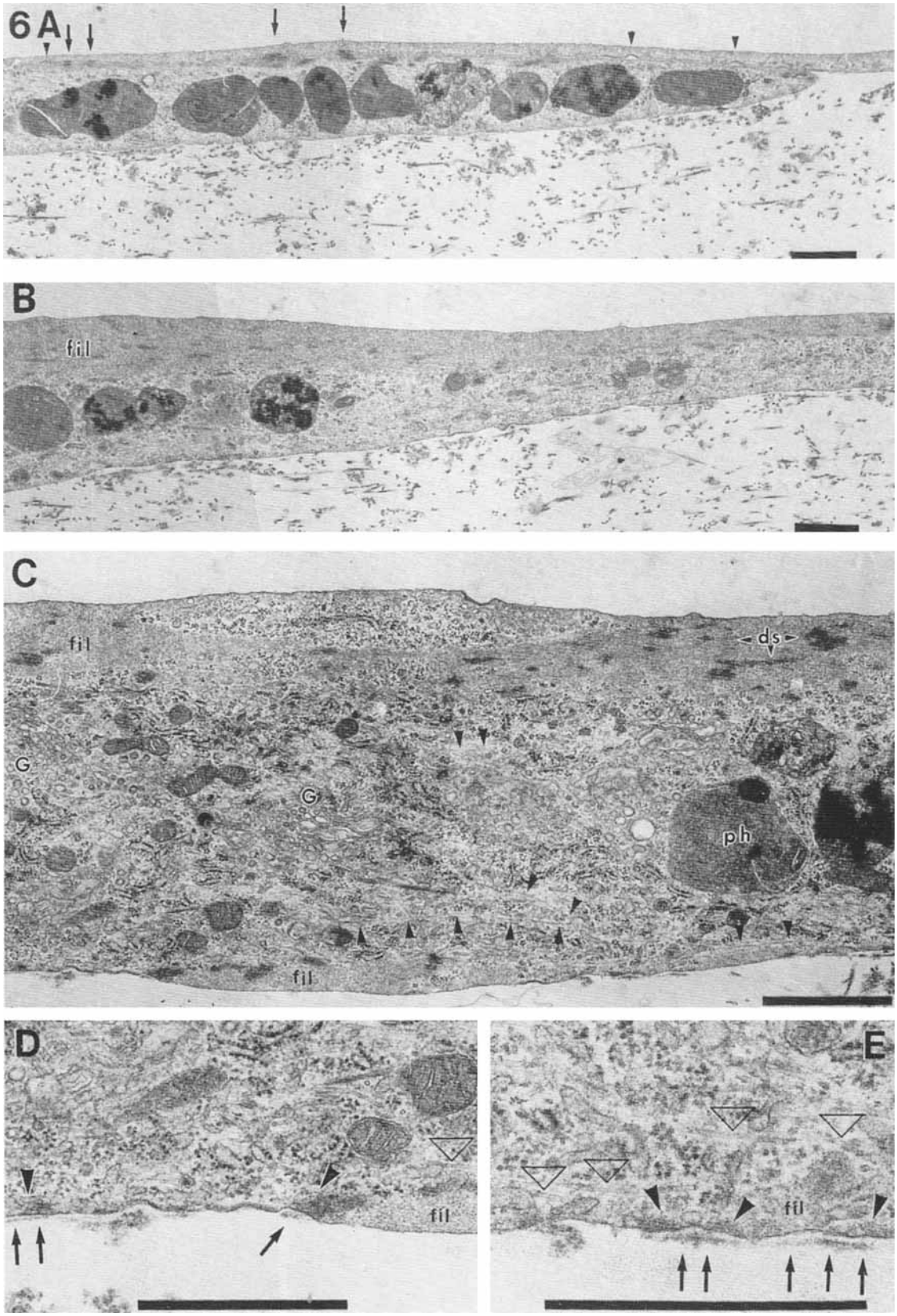

Fig. 6. Hepatocytes after 14 days in single gel culture. In **A,** the cells are remarkably flattened, completely devoid of microprojections on both sides, and just connected by desmosomes (arrows); coated pits (arrowheads). Inside the cytoplasm are large mitochondria and dense bodies. In **B** and **C,** thick layer of filaments (fil) with frequent dense spots (ds). In C, microtubules (arrowheads) are seen among ribosomes, a few cisternae of rER, phagosomes (ph) and Golgi complexes (G). In **D** and **E** (higher magnifications of lower cell border of C), discontinuous basement membrane material (arrows) opposed to dense intracellular areas (arrowheads) associated with filaments (fil) and microtubuli (open arrowheads). A, B ×12,000, C ×24,000, D ×40,000, E ×60,000, bar = 1 μm.

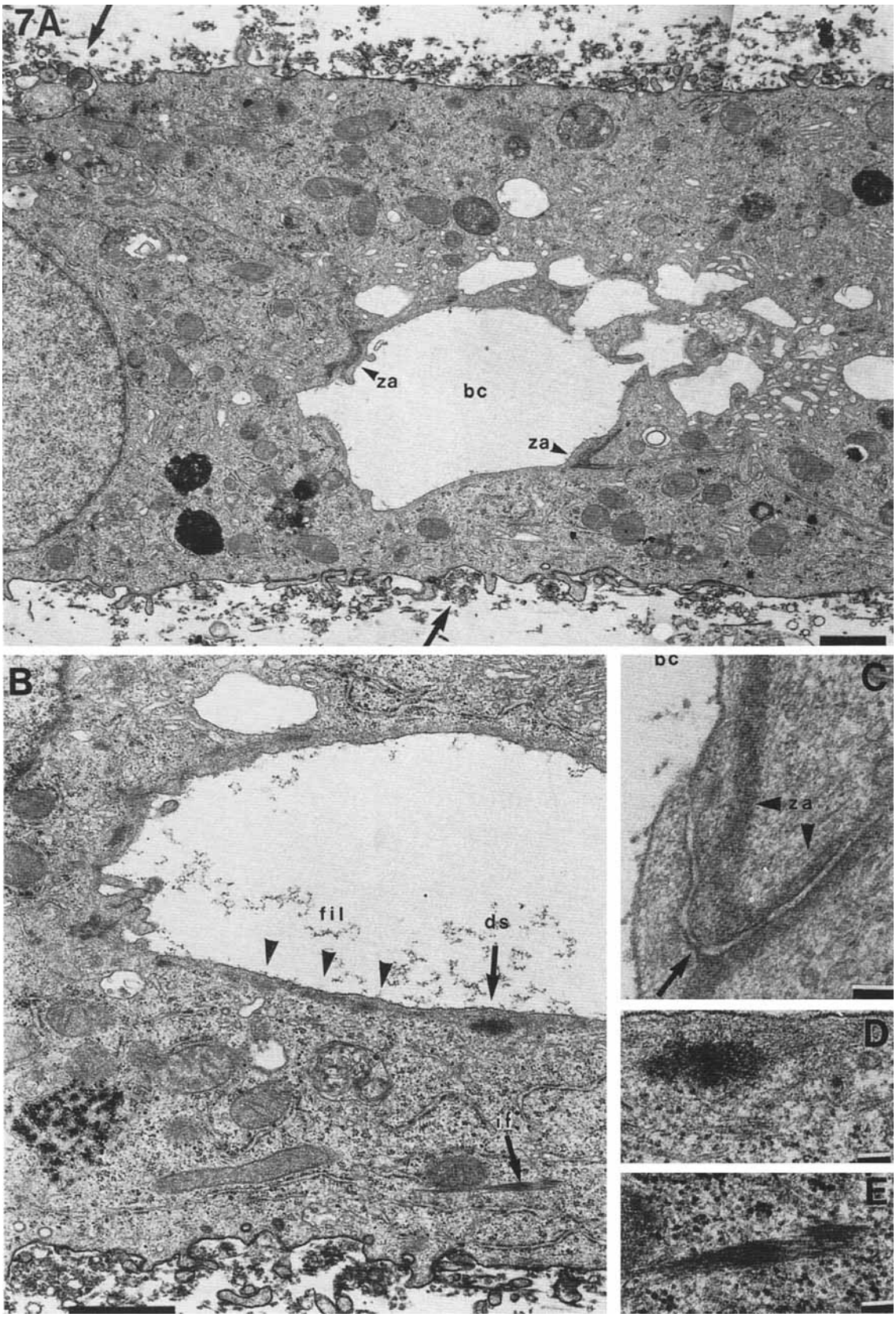

Fig. 7. Hepatocytes in double gel culture after 14 days. In **A,** the bile canaliculus-like expansion (bc) is cavernously enlarged and encircled by a distinct layer of filaments. Nearby the cytoplasm is occasionally filled with numerous vesicles, giving it a "foamy" appearance; zonulae adherentes (za). In **B,** the filaments (fil) with dense spots (ds), bundle of intermediate filaments (if); both structures (ds and if) can be clearly identified in **D** and **E**. In **C,** the junctional complex on the right side of the bile canaliculus (bc) of A, pentalaminar fusion of the adjacent plasma membranes (arrow) with an extensive zonula adherens (za). A ×12,000, B ×20,000, C ×80,000, D,E, ×60,000; in A,B bar = 1 μm, in C,D,E bar = 0,1 μm;.

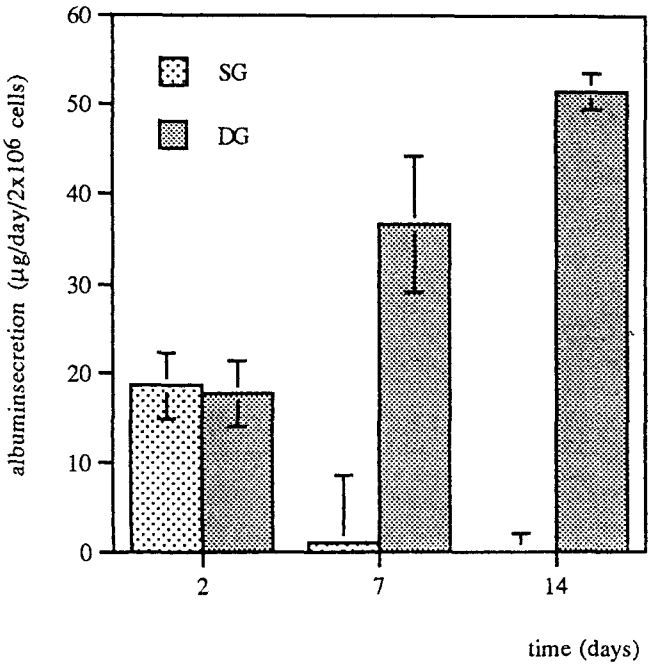

Fig. 8. Albumin secretion cumulated over 1 day in single and double gel system for the days 2, 7, and 14 in culture. After initial equivalence, there is rapidly declining secretion in single and steadily increasing one in double gel system.

they showed a more rapid dedifferentiation of the normal phenotype, i.e., a flattening and a general reduction of cell organelles, whereas elements of the cytoskeleton occurred in increasing amounts. In double gel, hepatocytes even after 14 days still had a basically normal morphology. Also, synthetic function as albumin secretion was preserved here, whereas it declined in the conventional culture system.

Bile Canaliculi and Intercellular Junctions

In situ the bile canaliculi are located at midway of the lateral surfaces of all the hepatocytes. Aspects of this distribution are evident in SEM-micrographs (see, e.g., Fawcett, 1994). In single gel culture, the expansions of the intercellular cleft were frequent but not among all the hepatocytes. This suggested that the bile canaliculi might not extend to the whole cellular circumference. In double gel cultures, the intercellular expansions were ubiquitary (after 2, 7, and 14 days) indicating that the network of bile canaliculi was completed, i.e., distributed as between hepatocytes in situ.

The intercellular expansions in both types of cell culture systems showed typical aspects of bile canaliculi as luminal microvilli, a junctional complex (zonula occludens, zonula adherens, and desmosomes). In contrast to hepatocytes in situ, the lateral cell surfaces were often atypical with narrow intercellular clefts all the way toward the sinusoidal surface and frequent interdigitating cytoplasmic processes. The order of the junctions was sometimes confused and junctional structures were seen not only close to the expansions as in situ (Chalcroft and Bullivant, 1970; Goodenough and Revel, 1970; Montesano et al., 1978; Jones et al., 1980) but interspersed over the whole lateral surface. An atypical localization of the tight junctions has been described in fetal liver explants (Montesano et al., 1978), indicating a loss of typical mature hepatocyte characteristics in culture in general.

The same suggestion also could be considered for the wide bile canaliculi-like expansions associated with sparse luminal microvilli. These are previously described in conventional single gel culture (Chapman et al., 1973; Wanson et al., 1977; Gebhardt and Jung, 1982) and in different situations such as embryological developing liver (Wood, 1965; DeWolf-Peeters et al., 1974), regenerating liver (Wood, 1965; DeWolf-Peeters et al., 1974), and bile duct obstruction (DeVos et al., 1975). The question arises whether the progressive enlargement of the bile canaliculi-like expansions represents an undifferentiated structure or a structural feature due to dilatation by an internal stasis of the secreted material. Since there are indications for a biliary secretion of hepatocytes in primary culture (Bonney and Maley, 1974; Bissel and Guzelian, 1975; Boyer et al., 1988), peribiliary bodies and peroxisomes, both involved in biliary secretion (Fahimi and Sies, 1987), are numerous, and the bile canaliculi-like expansions are closed by junctions, we suggest that the secretory product could be stored inside these bile canaliculi-like structures, causing their dilatation and the reported cytoskeletal alterations (see below). The absence of cavernous expansions in hepatocytes of single gel culture supports this assumption because there the secretory functions are declining with culture time (Guguen-Guillouzo et al., 1983; Bissel et al., 1987; Ben-Ze'ev et al., 1988; this study) and adjacent cells later remain mainly joined by desmosomes as seen here and elsewhere (Wanson et al., 1977; Gebhardt and Jung, 1982).

Organelles

The loss of specific hepatic functions in the conventional single gel culture cannot be characterized only by a reduction in transcription of liver specific genes (Bissel et al., 1987; Ben-Ze'ev et al., 1988), but also by a progressive and strong rarefication of the cell organelles as seen here. Other authors also reported rapid involution of cellular ultrastructure in single gel over a shorter time scale (2–5 days) than ours (Bissel et al., 1973; Chapman et al., 1973; Alwen and Lawn, 1974; Bernaert et al., 1977; Wanson et al., 1977; Gebhardt and Jung, 1982; Jung et al., 1982; Robenek et al., 1982). Compared to this, the hepatocyte organelles in the double gel culture are at least preserved up to 14 days, allowing intact albumin production and secretion. There is indication that the transcriptional regulation can be influenced by factors integrated into the composition of the extracellular matrix (Bissel et al., 1989) or by cell-cell contacts in coculture with other cell types (Guguen-Guillouzo et al., 1983; Mesnil et al., 1987). However, all these culture systems are more complex and require stimuli that in the double gel culture model do not seem to be necessary for intact cell differentiation.

The decline of the organelles in single gel culture is most pronounced for the mitochondria, which, although of inhomogeneous size with larger forms, were reduced in number until mostly lost after 14 days. In

the double gel up to 14 days, mitochondria had only moderate size inhomogeneity without apparent numerical changes. The phenomenon of mitochondrial enlargement and numerical reduction, as reported in conventional cultures (Chapman et al., 1973) and also observed after addition of glucocorticoides to the culture medium (Wiener et al., 1968; Berliner, 1975), possibly related with biochemical defects (Kimberg et al., 1968), is a clear indication of cellular dedifferentiation in the conventional culture form.

Other types of cell organelles (as rough and smooth endoplasmic reticulum, peroxisomes, phagosomes) and cell products (as glycogen or lipid) are seen in decreased amounts in the micrographs after 7 days and are almost lost after 14 days in single gel culture. This gradual deterioration of cellular morphology is responsible for and reflected by a parallel loss of albumin secretion as observed by ELISA.

Cytoskeleton

Cytoskeletal components in general, such as microfilaments, intermediate filaments, and microtubuli, increased in the cultured hepatocytes from day 2 to day 14 but in different locations. Assemblies of microfilaments (arranged as stress fibers) associated with bundles of intermediate filaments and accompanied at the intracellular side by numerous microtubules were observed. These were preferably expressed in the cell periphery underneath that plasma membrane bordering the medium (in single gel culture) or around the very large bile canaliculi-like expansions (in double gel culture at 14 days). Both represent locations that are in culture exposed to an unphysiological (possibly mechanical) stress, either due to surface phenomena (at the sinusoidal sides) or to biliary dilatation (at the apical sides). Our ultrastructural observations are supported by the finding of increased transcription rates of cytoskeletal genes (Ben-Ze'ev et al., 1988; Ezzell et al., 1993) and the immunohistochemical light microscopic identification of the respective cytoskeletal components in cultured rat hepatocytes (Ezzell et al., 1993). The "stress" on the hepatocytes seems to increase with duration of culture since the thickness of the respective filament layers also increases. The occurrence of dense spots inside the microfilament bundles may indicate the presence of contractile components.

An unsuitable culture environment in the single gel also may be indicated by the development of basement membrane-like deposits underneath the hepatocytes that are not observed in situ, even though extracellular matrix components are distributed in the space of Disse (Martinez-Hernandez, 1984). Atypical focal adhesion structures resembling a fibronexus (Singer, 1979) occur at these points connecting the cytoskeleton with extracellular material.

Plasma Membrane Differentiations

The influence of different culture techniques on hepatocyte differentiation is obvious also in the expression of plasma membrane differentiations, i.e., microvilli- and microplicae-like projections and coated pits and vesicles (indicating endocytotic processes) (Jones et al., 1980). These are usually found numerous on both sinusoidal cell faces of hepatocytes in situ (Bruni and Porter, 1965). Whereas the enclosure with rat tail collagen in double gel preserved intact hepatocyte polarity with normal cell surface differentiation, this disappeared in conventional single gel culture first from the "unphysiologic" face toward the liquid medium (even in techniques with a matrigel overlay on top of the hepatocytes) (Musat et al., 1993) and later also from the more "physiologic" face at the collagen side.

In conclusion, the double gel culture system provides distinctly better hepatocyte differentiation and metabolic function on the basis of a better preserved cellular ultrastructure than in conventional single gel culture. In the conventional single gel culture system, the hepatocytes undergo a rapid deterioration of the ultrastructure and rapid loss of at least one specific hepatocyte function. These differences occur using the same conventional extracellular matrix and medium in both systems.

Our findings hence show that for the hepatocyte, not only specific elaborated *compositions* of the matrix (hitherto extensively investigated, e.g., by Elsdale and Bard; 1972; Ben-Ze'ev et al., 1988; Schuetz et al., 1988; Caron, 1990) account for the cellular well-being but also, and probably equally important, the organization of the cellular microenvironment with respect to the *position* of extracellular matrix.

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